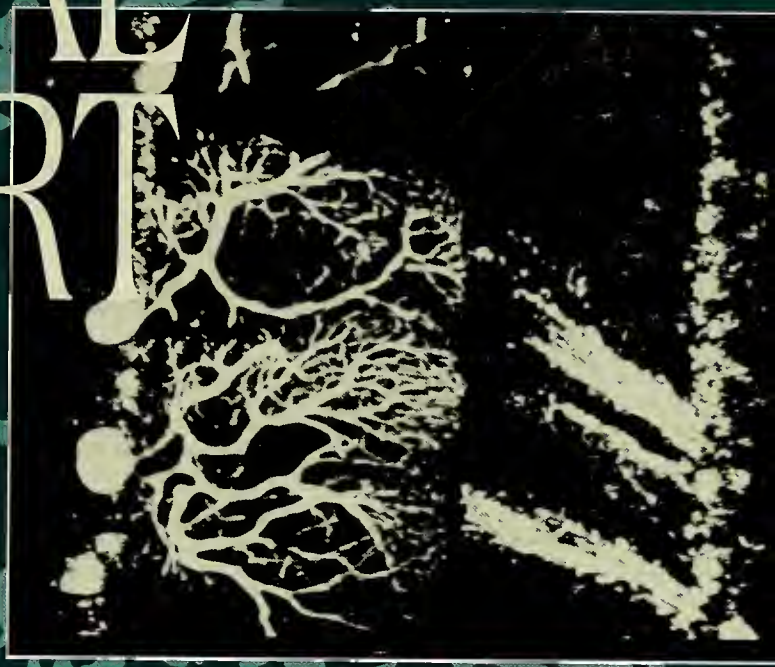
NATIONAL EYE INSTITUTE

ANNUAL REPORT



FISCAL YEAR 1993

Cover Photo

Immunofluorescence showing the Purkinje cells of a transgenic mouse brain reacting with a chloramphenicol acetyltransferase antibody. The transgene was driven by the promoter and enhancer of the chicken δ2-crystallin/argininosuccinate lyase gene. The photograph was taken by Dr. Steven Bassnet (Department of Anatomy and Cell Biology, Uniformed Services University of the Health Sciences) and is a 3-dimensional reconstruction using the Voxel View program on a Silicon Graphics Workstation taken with a confocal microscope. The photograph was originally published on the cover of *Developmental Dynamics* 196: 1993, and is described in Piatigorsky J: Puzzle of crystallin diversity in eye lenses. *Developmental Dynamics* 196:267, 1993.

National Eye Institute

Annual Report

Fiscal Year 1993

U.S. Department of Health and Human Services
Public Health Service

National Institutes of Health

Table of Contents

Statement of the Institute Director

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Carl Kupfer, M.D.

With the publication this year of our newest long-range plan *Vision Research—A National Plan: 1994-1998* we have taken stock of the accomplishments and current status of vision research and have focused once again on the exciting research opportunities that lie ahead. Our extramural and our intramural laboratories and clinical scientists have helped us make excellent progress in accomplishing our mission of conducting and supporting research, training, health information dissemination, and other programs relevant to eye diseases and vision disorders. About $235 million went to extramural researchers in the form of grant support and $5 million was expended to support research and development contracts. Another $7.2 million was used to support training awards. This funding has led to a number of important findings this year.

National Eye Institute (NEI)-supported researchers have demonstrated that mutations in several retina-specific genes cause photoreceptor degeneration in humans and mice. Although the mechanism by which these gene mutations lead to photoreceptor degeneration is unknown, scientists have suggested that there is a common pathway in the disease process. Apoptosis, or programmed cell death, appears to play a role in all of these retinal degenerations through fragmentation of the deoxyribonucleic acid (DNA) by intracellular enzymes at specific sites in the genome. If this is the case then there is the exciting possibility of an intervention for a variety of retinal degenerations based on inhibition of these DNA-cutting enzymes.

Our knowledge of the genetic loci for some of the macular degenerations has also been expanded. The genes for at least three forms of macular degeneration have been localized to specific chromosomes by NEI-sponsored investigators. A juvenile form of macular degeneration known as Best's disease has been mapped to chromosome 11, and two other forms of hereditary macular disease have been linked to chromosome 6.

Results from a prospective, double-masked clinical trial designed to assess the effectiveness of vitamin A and/or vitamin E supplements in halting or slowing the progression of retinitis pigmentosa (RP) were released. They showed that adults who supplemented their diets with 15,000 IU of vitamin A daily had on average about a 20 percent slower annual decline of remaining retinal function than those not taking this dose.

Last year, clinical trial results released by the Herpetic Eye Disease Study (HEDS) investigators showed that oral acyclovir was no better than a placebo in treating active herpes simplex stromal keratitis. Another part of the HEDS examined the effect of steroid eye drops as a treatment for this disease. This year, researchers conducting the study reported rapid improvement of stromal keratitis with immediate steroid therapy, but for those patients having their first episode of stromal keratitis, topical steroids could be safely deferred.

Progress has also been made in further understanding of the chaperon functions of α-crystallins. *In vitro* experiments have demonstrated that α-crystallin efficiently suppresses the aggregation of β- and γ-crystallins. This suggests that a biological role of α-crystallins is to prevent posttranslational changes in the interactions between lens crystallins and hence to maintain the transparent state of the lens.

NEI-supported scientists have been studying affected families in Michigan, the New England states, and Iowa in an attempt to identify a "glaucoma" gene and ultimately to characterize the protein encoded by this gene. Recently, the disease-associated gene has been mapped to chromosome 1. Although the link between juvenile onset glaucoma and the more prevalent primary open-angle glaucoma is unclear, finding the gene responsible for one form of glaucoma is a beginning in the quest for identification of at least one of this disease's causative factors.

The scientists conducting the Optic Neuritis Treatment Trial (ONTT) reported that a three-day, high-dose treatment with intravenous corticosteroids followed by a short course of oral corticosteroid reduced the rate at which study participants

developed multiple sclerosis (MS). Last year the ONTT scientists reported that this treatment enabled patients to recover their vision about two weeks sooner than would be the case without treatment but that oral prednisone, when used alone, was ineffective and actually increased a person's risk for future attacks.

Intramural scientists have continued their research efforts to understand the systems within the brain that process visual information and produce eye movements as well as to understand what happens when disease or trauma lead these systems to fail. Through the use of a superb animal model, these researchers have found that the animals respond to simulations of motion projected on a screen with postural changes similar to those reported in humans. This information will allow researchers to investigate further the regions of the brain that are known to process this type of visual motion information and to see whether alterations of these regions affect posture. They were also able to locate the approximate region of the frontal eye fields in humans and alter certain eye movements after first locating this region in monkeys. By using information obtained from animal models to locate areas in the human brain that perform similar functions, we may gain a better understanding of how the intricate mechanisms that guide the visual process operate normally and how they might be impaired by disease or injury.

In studies of the regulatory elements required for expression of genes in the eye and other tissues, intramural scientists have found that these elements are quite diverse with each having its own special properties. The elements may also be functionally redundant, that is, removing one does not necessarily eliminate the expression of the gene. A more complete understanding of gene expression in the eye may one day allow treatments to be directed to specific eye tissues.

Intramural researchers have continued their leadership role in the study of gyrate atrophy. Dietary intervention studies are continuing in families with two affected children. These studies have demonstrated a marked decrease in the retinal progression of this disorder in the children who began the dietary intervention at an early age. It is anticipated that this original work will lead to gene therapy aimed at preventing this disease.

Intramural epidemiologists investigated the effect of vitamin and/or mineral supplements on the risk of developing age-related cataracts in conjunction with two National Cancer Institute (NCI) trials using the same vitamin and/or mineral interventions in a population with chronic nutritional problems and high rates of esophageal and stomach cancer. In these highly cost-effective studies of populations with chronic deficiencies of multiple nutrients, NEI investigators and their colleagues found that use of the supplements was associated with a decreased risk of nuclear cataract. Additional research is underway to determine whether these findings apply to less nutritionally deficient populations.

These are only a few highlights of the important accomplishments of vision researchers in fiscal year (FY) 1993, a year in which we marked the 25th anniversary of the establishment of the NEI. Anniversary activities were organized around the theme A Celebration of Vision Research and were designed to provide the American public with a report on its investment in vision research. A traveling science museum has been developed that demonstrates the progress and accomplishments of vision research during these past 25 years.

It is with great sadness that we must also note the passing of our friend and colleague Julian M. Morris. Much of what we have accomplished, since his selection as the NEI's first information officer in 1970, we owe to him and his dedication to the field of vision research. In recognition of his many contributions, we have dedicated *Vision Research—A National Plan: 1994-1998* to his memory. We will miss him.

Extramural Research

Report of the Associate Director for Extramural Research

Jack A. McLaughlin, Ph.D.

Research activities supported by the Extramural Vision Research Program address the leading causes of blindness and impaired vision in the United States, including retinal diseases, corneal diseases, cataract, glaucoma, strabismus, and amblyopia. The program seeks to increase understanding of the normal development and function of the visual system; to understand the causes of and better diagnose, prevent, and treat sight-threatening conditions; and to enhance the rehabilitation, training, and quality of life of individuals who are partially sighted or blind.

In working to this end, the Vision Research Program supports vision research through grants, cooperative agreements, and research and development contracts; encourages high-quality clinical research, including clinical trials and other epidemiologic studies; encourages research training and career development in the sciences related to vision; sponsors scientific workshops in high-priority research areas to encourage exchange of information among scientists; and carries out a construction, alteration, and instrumentation program of grants for public and private nonprofit vision research facilities.

For FY 1993, an estimated total of $235,005,000 was expended for NEI extramural grants, cooperative agreements, and research and development contracts in the following categories and amounts:

Research Grants	$222,735,000
Research Training Awards	$7,226,000
Research and Development Contracts	$5,044,000
Total Extramural Support	$235,005,000

Concurrent with the reorganization of the NEI, a number of personnel changes occurred in the Extramural Program during this fiscal year. Among these were the appointments of Drs. Loré Anne McNicol, Peter A. Dudley, and Richard L. Mowery, as director of the division of extramural activities, as director of the division of basic vision research, and as director of the division of collaborative clinical research, respectively.

The following sections highlight some of the recent accomplishments of the NEI-supported investigators.

Division of Basic Vision Research

Peter Dudley, Ph.D., Director

Retinal and Choroidal Diseases

Retinal Degeneration and Apoptosis

Cell death is an important part of normal developmental programs. For example, the balance of cell survival with neuronal cell death is thought to be an important mechanism whereby an organism controls the interconnections between populations of developing cells. Apoptosis, or programmed cell death, seems to be an important mechanism used by the retina during its development into a functional layered tissue.

Mutations in several retina-specific genes have been shown to cause photoreceptor degeneration. Mutations in the rd, rds/peripherin, and rhodopsin genes cause retinal degenerations in humans and mice. The mechanism by which these gene mutations lead to photoreceptor degeneration is unknown. Recent work by Fulton Wong at Duke University shows that although the phenotypes of these degenerations are different, there appears to be a common pathway in the disease process. Apoptosis appears to play a role in all of these retinal degenerations through fragmentation of DNA by cleavage at specific sites in the genome. Apoptosis has been demonstrated to occur in retinal degenerations by observing a characteristic "ladder" of DNA fragments using gel electrophoresis. The

DNA ladder gel pattern results from a predictable fragmentation of genomic DNA at internucleosomal sites during the degeneration process.

Although apoptosis may be a common pathway that leads to cell death, the mechanism triggering this process is unknown. It seems likely that intracellular enzymes called endonucleases are activated to cut DNA into small fragments. If this is the case, then there is the exciting possibility of an intervention for a variety of retinal degenerations based on inhibition of these DNA-cutting enzymes.

Cancer Associated Retinopathy

Disorders of the retina leading to a loss in vision can result from the remote effects of cancer. Certain types of tumors at a distant site can set up an immunological reaction that can manifest itself in the retina as well as in other tissues of the nervous system. When the retina is involved in this paraneoplastic syndrome, it is called cancer-associated retinopathy or CAR. This syndrome develops mainly as a result of a primary tumor in the lung, although other tissues may be implicated. Metastasis is not involved, but rather molecules of the immune system called autoantibodies appear in the serum of patients. This immunological reaction appears to be, in part, a response of the host or patient to the tumor. Thus, the patient appears to be producing antibodies as a defense against the tumor in the hopes of limiting its growth. Vision loss is frequently the first sign of illness leading to subsequent clinical examinations that identify the causal cancer.

Patients experiencing CAR report a sudden loss of vision resulting from inactivation of important proteins in the retina by the autoantibodies generated in response to the tumor. Patients with other types of retinopathies do not produce antibodies that react with the CAR protein or antigen in the retina, indicating a high degree of specificity of the immunological reaction.

Several NEI-supported investigators are looking into the role of specific retinal antigens in the development of CAR in patients, and further work hopefully will uncover the basis of this disease. Recent work has shown that one protein involved in phototransduction, called recoverin, may be present in cancer cells. It is the immune response to this protein that results in retinal involvement. Further, although recoverin has been found only in rod cells, antisera to recoverin label both rods and cones. The reason may be that there is a molecule in cones that has some sequence homology to recoverin and that reacts with antirecoverin antibody. Current investigations are focusing on the isolation and cloning of the human recoverin gene. The mouse gene has recently been cloned, and the deduced amino acid sequence is identical to that of human recoverin protein. Segregation analysis shows close linkage to the tumor suppressor gene p53. The current hypothesis being tested is that CAR is the result of a single mutational event in a cell that deletes the tissue-specific regulatory elements of the recoverin gene while joining its coding sequence to an active gene. This would delete the function of the p53 cancer suppressor gene and simultaneously turn on synthesis of recoverin. The cell becomes cancerous because the p53 protein product is either absent or inactive and no longer functions as a cancer suppressor.

These kinds of studies could lead to a method for early diagnosis of cancer and the opportunity to institute treatment at an early stage.

Molecular Genetics of Macular Degeneration

Macular degeneration is the most common cause of severe visual impairment in older persons in the United States. It robs otherwise healthy older Americans of useful vision, depriving them of the ability to read, drive, and enjoy leisure activities. Currently, there is no effective treatment for the vast majority of individuals with this condition because the basis for the disease is not understood.

The genes for at least two forms of macular degeneration have been localized to specific chromosomes by Dr. Richard Stone at the University of Iowa. Best's disease is an autosomal dominant condition characterized by the accumulation of lipofuscin within and beneath the retinal pigment epithelium (RPE). It has an earlier age of onset than the more prevalent age-related macular degeneration (AMD), and there is an absence of drusen. Drusen are deposits of extracellular material lying between the RPE and Bruch's membrane. The Best's gene has been localized to chromosome 11q13. Dominant macular dystrophy with flecks (DMDF) is an autosomal recessive degeneration characterized by severe vision loss with macular lesions ringed with

fleck-like deposits of yellow pigment. Preliminary evidence indicates that the gene for DMDF is localized to chromosome 6q.

Linkage analysis offers the opportunity to map human disease genes when the causative agent is unknown, as is the case for macular degenerations. Gene mapping can lead to actually cloning the gene responsible for specific eye diseases, for example, AMD, by application of reverse genetics.

Retinitis Pigmentosa

RP is a group of hereditary eye diseases with an overall incidence of about 1 in 3,500 births in the United States. The emotional and economic costs of the disease to society are enormous, particularly because no effective treatments are known for most types of retinal degeneration. RP is genetically heterogeneous and can be transmitted as a dominant, recessive, or X-linked trait.

NEI-supported research has led to significant advances in identifying the molecular defects in different forms of RP. Scientists reasoned that a gene coding for a structural or a functional protein, important in the physiology of the pigment epithelial and the photoreceptor cells of the retina, might be defective in patients with retinal degenerations. With this candidate gene approach, it was discovered that 20 to 30 percent of individuals with autosomal dominant RP have a mutation in the rhodopsin gene. In autosomal recessive RP, a different rhodopsin gene mutation was shown to be present. Mutations have also been found in the human homologue of the murine rds locus, the photoreceptor-specific peripherin/RDS gene, in some families with autosomal dominant RP. Although the function of this protein is not known, it may serve as an adhesion molecule, stabilizing the outer segment discs through interactions across the intradiscal space.

Most recently, Dr. Ted Dryja at the Harvard Medical School has found that a third photoreceptor-specific gene is defective in patients with another form of autosomal recessive RP. Mutations in the gene encoding the beta-subunit of the cGMP phosphodiesterase, a key molecule of the visual transduction pathway, are present. This is of particular interest because the murine homologue of this gene is defective in the *rd* strain of mice with retinal degeneration.

In related research, a two-base pair deletion was found in the human peripherin/RDS gene in a family with autosomal dominant retinitis punctata albescens, an uncommon form of retinal degeneration clinically related to RP. A defect in the rhodopsin gene was also found in congenital stationary night blindness. In studying the molecular mechanism of this genetic defect Dr. Dryja's group found that the mutant rhodopsin protein activated transducin without binding its natural chromophore, retinal. This appears to be caused by abnormal constitutive activation of transducin in the phototransduction pathway.

The identification of genetic defects in retinal degenerations and dystrophies is an important step in developing effective therapeutic strategies. With this information in hand, scientists will now be able to explore the molecular mechanisms responsible for these diseases and translate this information into rational and effective diagnosis, treatment, and prevention strategies.

Corneal Diseases

The corneal stroma is unique among the collagenous connective tissues in being transparent. Understanding the molecular basis of transparency requires a detailed knowledge of the regulation of the collagen types, proteoglycans, and glycoproteins expressed in this organ. The Corneal Diseases Program is supporting several laboratories engaged in studies of the development and synthesis of specific collagen genes.

Dr. Bjorn R. Olsen at Harvard Medical School has been investigating the synthesis of type VIII collagen, one of the short-chain collagen species. This protein had been reported as a component of the intima layer of vascular endothelium and as the major structural component of Descemet's membrane in the cornea. Dr. Olsen has cloned and sequenced the two genes encoding type VIII collagen and has found that it exists as a heterotrimer of composition $[\alpha 1(VIII)]_2[\alpha 2(VIII)]$. *In vitro* hybridization studies revealed the surprising observation that the $\alpha 1$ gene is expressed in the lens as well as in the cornea. Chromosomal localization studies have shown that the $\alpha 2$ gene is located at the region of the defect dysgenetic lens (dyl) gene. This is a recessive hereditary disorder that shows a persistent connection between the lens and the corneal epithelium as well

as various degrees of corneal opacity. Dr. Olsen has prepared transgenic mice carrying defects in both the α1 and α2 genes. This should permit a more detailed examination of the unexpected role of type VIII collagen in the embryonic development of the cornea and lens.

Lens and Cataract

Lens Biochemistry

The α-crystallins are major structural proteins of the vertebrate lens and contribute to its refractive mass and transparency. During the past decade, it has been shown that in some species "housekeeping" enzymes that are found in nonlenticular tissue are recruited to serve as structural crystallins in the lens. This has led to the concept of "gene-sharing" implying that a single gene encodes a protein with dual functions. Evidence for a dual function for α-crystallins has come from two lines of investigation. First, the α-crystallins are found in nonlenticular tissues, including the heart, lung, spinal chord, brain, kidney, and retina. αB-crystallin specifically accumulates in many neurological disorders. Second, the α-crystallins are structurally related to the family of heat-shock proteins that can be induced by heat or hypertonic stress and accumulate in a number of pathological conditions.

These two lines of evidence have converged with the recent finding by Dr. Joseph Horwitz from the University of California at Los Angeles that α-crystallins function *in vitro* as molecular chaperons. These are a subset of heat-shock proteins that are overproduced in response to physiologic "stress" and that act by affecting protein-protein interactions. They stabilize native protein conformations, mediate the folding and correct oligomeric assembly of nascent proteins, catalyze the membrane transloctions of secretory proteins, and prevent protein aggregation under conditions of heat denaturation. *In vitro* experiments have demonstrated that α-crystallin efficiently suppresses the aggregation of β- and γ-crystallins. This suggests that a biological role of α-crystallins is to prevent posttranslational changes in the interactions between lens crystallins and hence maintain the transparent state of the lens.

Developmental Biology

In many vertebrate species, proper iris and cornea development appears to be coupled to lens growth and viability. A new tool in the study of early lens development has been the *small eye* (*Sey*) mutation in mice in which abnormalities in lens development are accompanied by other anterior chamber defects. This mutation has resulted in a potentially useful animal model of aniridia. This condition results from defects in PAX-6, a gene encoding paired-box and homeobox motifs that are expressed in the developing eye. It shares homology with paired box genes of *Drosophila* that control the development of body segmentation. The homeobox encodes the helix-turn-helix motif seen in DNA-binding proteins.

Aniridia is a human developmental disorder closely related to the *Sey* mutation and is characterized by hypoplasia of the iris and is commonly associated with other clinical anomalies such as cataracts and lens dislocation. The disease is inherited in an autosomal dominant fashion. It is frequently cotransmitted with Wilms' nephroblastoma, genitourinary abnormalities, and mental retardation (termed WAGR complex), demonstrating a close linkage with the genes responsible for these anomalies. This has greatly facilitated the mapping and identification of the human aniridia gene.

The WAGR complex has been mapped to a large interstitial deletion on the short arm of human chromosome 11. Dr. Lisa Davis at the Applied Genetics Laboratory in Melbourne, Florida and Dr. Richard Maas of Harvard University have been working independently to fine map and characterize the gene. This area is homologous to the region of the mouse chromosome 2, which contains the *Sey* gene. Using a mouse PAX-6 clone as a probe, the aniridia region has been identified in human cDNA libraries. Physical mapping of the aniridia gene using DNA isolated from patients with aniridia will provide us with new insights into ocular development.

Glaucoma

Molecular Genetics

Glaucoma is a potentially blinding condition associated with increased intraocular pressure (IOP) and gradual destruction of the optic nerve. Little is known about the underlying causes of glaucoma and the accompanying nerve degeneration that leads to loss of vision. Furthermore, the relative influence of genetic and environmental characteristics is poorly understood. Fortuitously, juvenile onset glaucoma, a form of the disease characterized by early adulthood onset and elevated IOP, displays an autosomal dominant pattern of inheritance. The unambiguous phenotype, high degree of penetrance and early age of onset, makes a genetic approach ideal for the study of this form of the disease.

Dr. Julia Richards at the University of Michigan has been working with a number of families that have sufficient meiotic events to perform genetic linkage studies. To date, family histories have been collected from families in Michigan, the New England states, and Iowa. The ultimate goal is to identify a "glaucoma" gene using positional cloning, followed by sequencing analysis to characterize the encoded protein. Recently, the disease-associated gene has been mapped to chromosome 1. Corroborating data from different laboratories using different families have confirmed this locus. In one of the Michigan families, linkage analysis has placed the gene within a 14 centimorgan region of the chromosome, at 1q21-q31.

The link between juvenile onset glaucoma and primary open-angle glaucoma is unclear, but finding the gene responsible for one form of glaucoma is a beginning in the quest for identification of at least one causative factor.

Ganglion Cell Function

Currently, there is controversy about whether glaucomatous damage is selective for a subset of ganglion cells. Each area of the retina has several functionally distinct types of ganglion cells serving the same photoreceptor cell in parallel pathways. The majority of ganglion cells have large cell bodies and large dendritic fields and are classified as M or magnocellular. The P or parvocellular ganglion cells are more numerous, have small cell bodies, restricted dendritic fields, and are involved in color vision. Early investigations seemed to indicate that glaucoma initially damages the large diameter nerve fibers that are prevalent in the M pathway.

More recently, psychophysical studies carried out by Dr. Chris Johnson from the University of California at Davis indicate that early neuropathy involves both P and M pathways. Using longitudinal studies, he has shown a link between abnormalities detected using blue-on-yellow perimetry and temporal modulation perimetry. Temporal modulation perimetry is a noninvasive psychophysical technique used to assess visual sensitivity at various frequencies of flickering light. Short wavelength light responses are diagnostic of the P pathway that processes spatial and finely detailed information. Flicker sensitivity at low frequencies is a good monitor of the M pathway. Blue-on-yellow perimetry has application for psychophysically isolating and measuring the sensitivity of the short-wavelength or blue cone pathway. Johnson's work suggests that there may be a lack of selectivity for any particular ganglionic cell subset in glaucoma. Patients tested with blue-on-yellow perimetry show deficits that precede standard visual field defects. Using temporal modulation perimetry there was an overall loss of flicker contrast sensitivity in patients with early glaucomatous visual field loss, but this deficit was not selective for high frequencies. The decrease in sensitivity demonstrated in these tests occurs at the same time that early visual field defects are seen. This is consistent with the idea that early glaucomatous damage is not limited to a specific subset of ganglion cells.

Ultimately, understanding optic nerve pathology can lead to more sensitive predictors for the risk of glaucomatous nerve fiber and hence vision loss.

Aqueous Humor Dynamics

An important role of the aqueous humor in the anterior portion of the eye is to maintain the proper IOP. Aqueous humor is derived from plasma in the capillaries that feed the anterior portion of the eye via a specific tissue—the ciliary body epithelium. Fluid produced by the epithelial cells (inflow) leaves the eye via the trabecular meshwork and Schlemm's canal (outflow), reentering the vascular system

through the venous route. It is the balance between inflow and outflow that maintains the IOP. A major aim of current glaucoma research is to gain a better understanding of the mechanisms that regulate these two pathways.

Our understanding of outflow biology has been enhanced by a recent discovery by the laboratory of Dr. James Nathanson at Massachusetts General Hospital, showing that nitrovasodilators lower IOP. Nitrovasodilators are a class of compounds produced in response to increased levels of nitric oxide in the cell. Nitric oxide has been shown to be an important mediator in many physiological functions, including muscle relaxation, vasodilation, and transmission of neural impulses. All these effects are mediated by the second messenger cGMP. This finding adds important information to our understanding of outflow regulation and opens the door to the possibility of new therapeutic strategies.

Strabismus, Amblyopia, and Visual Processing

Development of Visual Pathways

One of the primary characteristics of the visual system is the precise pattern of connections, a virtual map, that exist between the retina and the visual centers of the brain. This pattern is established during embryonic development and refined during early life. Activity mediated by chemical signals (neurotransmitters) at the contact points (synapses) between nerve cells is suspected to play an important role in this developmental process. Research by Dr. Steve McLoon at the University of Minnesota has shown that the presence of the chemical precursors for nitric oxide, a recently discovered neurotransmitter, in a visual center of the brain coincides with the timing of the ingrowing processes from the retina in the chick embryo. In fact, the concentration of these chemicals reaches a peak just as the initial visual map is being established by the terminals of the retinal cells. Unlike the results from other studies that have implicated a number of chemical signals in the establishment of visual maps in the developing nervous system, these results demonstrate the presence of a chemical signal at the right time and the right place needed to establish a precise map.

In related research, Dr. Carla Shatz from the University of California at Berkeley finds that when ganglion cells in the retina first make their connections with nerve cells in the brain, the pattern is not nearly as precise as it is in the adult. Many extra connections are made that are later pruned away. How do cells "know" which connections to maintain and which ones to eliminate? Dr. Shatz examined all branches to determine if electrical signals were being transmitted from one nerve cell to the next. Branches to be eliminated were found to function while they were present. This result lead Dr. Shatz to envision that perhaps a branch from a nerve in the eye confirms that it is in the correct location by sending a signal to the nerve cell in the brain to verify its location, much like placing a telephone call to verify an address. Dr. Shatz found that by blocking the signaling of the nerve cell it is possible to stop incorrect branches from being removed. Where do these signals originate? The connections between the eye and the brain form very early in fetal life, even before vision begins. Dr. Shatz suspected that the nerve cells in the eye might be signaling spontaneously to nerve cells in the brain. Dr. Shatz discovered that the retinal ganglion cells are spontaneously and repeatedly signaling their target cells in the brain during the weeks before vision takes over. Thus, in the visual system, and very likely elsewhere in the developing brain, nerve cell signaling before birth plays a crucial role in establishing correct connections.

Plasticity in the Visual Cortex

The traditional view states that the overall architecture and the connections between nerve cells in the adult visual cortex of the brain are fixed following a period in early postnatal life when these connections can be modified. This early period is called the critical period, and the ability of nerve cells to modify their connections is called plasticity. The connections between nerve cells carry visual information encoded in signals that are transformed and integrated as they ascend sequentially through visual centers in the brain. The transformation that occurs in these centers is analyzed in terms of receptive fields. These are sets of nerve cells that encode features of a visual stimulus falling on the retina and connect with other sets of visual nerve cells along the visual pathway. In this way receptive

fields form the building blocks that underlie visual perception.

In the adult, the traditional view holds that the cortex processes the visual scene through a fixed set of receptive fields, handing on information to the next stage in the visual pathway. Clearly, our ability to store new memories in adulthood requires some form of cortical plasticity, but it was generally believed that this would only occur in high-order association.

Experiments by Dr. Charles Gilbert at the Rockefeller University have changed our view of cortical visual processing. Dr. Gilbert made small, focal retinal binocular lesions in monkeys and found that the area of cortex receiving input from those parts of the retina became initially silenced, as expected. However, to his surprise, in adult animals the initially silent area of the cortex becomes remapped, responding instead to areas outside the lesion. Even more surprising was the finding that quite striking changes could be observed physiologically within minutes after making the lesion, and, finally, Dr. Gilbert found that a lesion is not necessary, certain patterns of visual stimulation can cause receptive fields to expand and contract over a time scale of minutes.

The finding of this degree of cortical plasticity in adult animals presents a radically different idea of how the cortex works, and the functional implications of the findings are closely related to the time course over which the changes take place. Dynamic changes over a time scale of minutes are useful for adapting to changes in the sensory environment. It is as if the cortex is constantly expanding and contracting its representation of various aspects of the sensory environment in response to the amount of input it receives from particular sets of stimuli. Future studies may uncover how these changes in visual cortex relate to learning and memory such as the representation of complex images occurring in higher cortical areas.

Development of Myopia

More than 25 percent of the adult population of the United States is near-sighted (myopic). This refractive error usually develops in the vast majority of people between the ages of six and 14 years. The relationship between accommodation and the development of myopia has been a controversial topic for a number of years. Animal models of myopia are beginning to shed some light on this issue. Recent experiments in the tree shrew (a mammal closely related to primates), the chicken, and the monkey have shown that a biological feedback mechanism controls the shape of the eyeball. Under normal circumstances this effect causes the focal length of the visual image to fall on the retina (i.e., the eye is in good focus). Dr. Thomas Norton from the University of Alabama at Birmingham has done experiments that indicate that the presence of visual images on the retina produces a signal within the eye that, through a cascade of events, affects the structural nature of the sclera, the outer coat of the eye, without involving the central visual nervous system. Myopia occurs when this mechanism is disrupted, by visual deprivation in animals and by unknown perturbations in humans, causing the eye to become too long for its focal length. Work by Dr. Norton suggests that blurred images, or form deprivation, slow the accumulation of proteoglycans and collagen, two chemical components of the sclera. This in turn may cause the sclera to be less resistant to IOP and consequently causes the eye to become too long, producing myopia.

Low Vision

AMD of the retina is a leading cause of low vision and poses a particularly difficult problem for vision testing because of the central field scotomas (blind areas) that commonly result from this disorder. Recent developments in eye monitoring technology have made it possible to position a target very precisely on known locations of the retina. This has great potential for both documentation of visual loss and possible retraining of eye movements to enable use of remaining intact parts of the retina.

Progress has been made in developing new devices that aid and assist visually impaired persons, e.g., more ergonomically satisfying magnifiers, cosmetically acceptable telescopic spectacles, and voice control and output for computers. Attention now is focused on devices to assist in changes in terrain, text navigation for both printed materials and computer screens, image processing, and route-finding.

Division of Collaborative Clinical Research

Richard Mowery, Ph.D., Director

The Division plans and directs a program of grant, cooperative agreement, and contract support for applied clinical vision research, including clinical trials, natural history studies, surveys, cohort studies, and studies of cases and controls. The Division manages 21 clinical trials, 11 epidemiology studies, and three eye health education demonstration projects with an annual budget of $38.7 million.

Research Results

Retinitis Pigmentosa

RP is a diverse group of hereditary retinal diseases that cause a progressive degeneration of the rod and cone photoreceptors and loss of visual function. RP affects approximately 100,000 people in the United States and approximately 1.5 million people worldwide. Individuals with RP typically begin to lose peripheral vision in adolescence and early adulthood, and most lose central vision later in life. Results from a prospective, double-masked clinical trial designed to assess the effectiveness of vitamin A and/or vitamin E supplements in halting or slowing the progression of RP showed that adults who supplemented their diets with 15,000 IU of vitamin A daily had on average about a 20 percent slower annual decline of remaining retinal function than those not taking this dose. Based on this finding, an average patient who started taking a 15,000 IU vitamin A capsule at age 32 would retain some useful vision until age 70, whereas a patient not on this dose would lose useful vision at age 63. The study also found that in patients taking high-dose vitamin E supplements the disease appeared to progress faster on average than in patients taking a trace amount of the vitamin.

Cytomegalovirus Retinitis

Cytomegalovirus (CMV) retinitis is a potentially blinding disease of the retina that affects about 25 percent of people with acquired immunodeficiency virus (AIDS). NEI supports a network of investigators with expertise in AIDS clinical research, retinal diseases, and clinical trial methodology to expedite the testing of treatments for CMV retinitis and other ocular complications seen in patients with AIDS. This network is called the Studies of Ocular Complications of AIDS (SOCA). The first clinical trial conducted under SOCA was designed to compare the efficacy and safety of foscarnet and ganciclovir. The investigators found that patients treated with foscarnet lived longer than those who received ganciclovir. Foscarnet patients lived an average of 12.6 months after starting treatment compared with 8.5 months for patients taking ganciclovir. The drugs appeared to be equally effective in halting the progression of CMV retinitis and preserving vision.

Vitreoretinopathy

The most common cause of failure in retinal detachment surgery is the development of abnormal contractile tissue on the retinal surface. Mild forms of this condition can sometimes be treated by external surgery and the retina successfully reattached. However, in more severe forms intraocular surgery is required. The Silicone Oil Study, a multicenter clinical trial, was designed to evaluate the benefits and risks of using a long-acting gas or silicone oil as an aid in reattaching the retina. The study found that use of silicone oil is superior to use of long-acting gas, resulting in a higher rate of successful retinal reattachment.

Retinopathy of Prematurity

More than 4,000 infants weighing less than 1,251 grams at birth underwent sequential ophthalmic examinations to monitor the incidence and progression of retinopathy of prematurity (ROP) in the multicenter Cryotherapy for Retinopathy of Prematurity Clinical Trial. Two-thirds of the infants developed some degree of ROP. The incidence and severity of ROP were higher in lower birth weight and gestational age categories. African-American infants appeared less susceptible to ROP than did Caucasian infants.

Herpes Simplex

About a one-half million Americans are affected by ocular herpes, which often begins as a relatively painful sore on the surface of the cornea. Like herpes cold sores, these ocular lesions may

periodically recur, and the herpes virus can also over time cause an inflammation deep inside the cornea. This advanced infection is known as herpes simplex stromal keratitis, which can lead to severe corneal scarring, inflammation of the interior of the eye, and even blindness. A randomized, controlled clinical trial was conducted as part of the HEDS to evaluate whether oral acyclovir, when given to patients with steroid and antiviral eye drops, improved the treatment of active herpes simplex stromal keratitis. Researchers randomly assigned 104 patients to take either oral acyclovir or a placebo. After a 10-week treatment regimen and a six-month followup period, oral acyclovir was found to be no better than a placebo in successfully clearing the stromal keratitis. This indicates that the financial cost and minimal potential health risk associated with the use of this drug is not warranted.

The role of acyclovir in the prevention of recurrences of herpetic eye diseases during the course of one and one-half years and the role of acyclovir in preventing progression of superficial (epithelial) keratitis to the more severe stromal keratitis, or iritis, is currently being examined by the HEDS research group.

Although many ophthalmologists use steroids to control the corneal inflammation associated with herpetic stromal keratitis, clinical research has yielded mixed results regarding their overall effect. For example, some clinicians have reported encouraging results with this treatment, although others have indicated that steroid therapy worsens or prolongs the corneal lesions and predisposes patients to known complications such as glaucoma and cataract. A second randomized, clinical trial conducted as part of the HEDS examined the effect of steroid eye drops as a treatment for active herpetic stromal keratitis. After 10 weeks of treatment and six months of patient followup, corneal inflammation was held in check longer and corneal inflammation cleared faster in patients treated with steroids. However, delaying steroid therapy by one-to-three weeks did not significantly influence lesion recurrence or affect visual acuity at six months. Thus, rapid improvement of stromal keratitis was achieved with immediate steroid therapy, but for those patients having their first episode of stromal keratitis topical steroids could be safely deferred.

Corneal Transplantation

More than 40,000 corneal transplant operations are performed annually in the United States. But about one in 10 patients receiving a corneal transplant is at high risk of rejecting the donor tissue or graft because: (1) they have previously rejected a corneal transplant or (2) new blood vessels have grown into their damaged cornea, introducing immune cells into this normally avascular region of the eye that may later recognize the graft as foreign and attack it.

The Collaborative Corneal Transplantation Study (CCTS) was designed to evaluate whether donor-recipient tissue typing, transplanting a donor cornea that has cell-surface proteins (human leukocyte antigens [HLA]) that closely resemble those on the recipients's natural cornea, helps to prevent transplant rejection. These antigens serve as molecular "fingerprints" on every cell in the body and allow a person's immune system to distinguish its own cells from those belonging to another person. Previous studies had suggested that closely matching the donor's HLA with those of the recipient might increase the likelihood that the immune system would accept, rather than reject, the donor tissue.

After three years of patient followup, CCTS researchers found that people who received corneal transplants with well-matched antigens did not fare significantly better than those with a poor match. Each patient group had similar rates of initial immune reactions, graft rejection, and graft failure due to infection or other causes. These findings indicate that tissue typing was not an important factor in transplant survival.

If donor-recipient tissue typing were to become standard practice in corneal transplantation, it would greatly increase the cost and waiting period for this operation. The process of matching antigens is labor intensive and would add at least $1,000 to the nearly $5,000 cost of a corneal transplant operation. Moreover, because there is already a national shortage of donor corneas, high-risk patients would likely have to wait even longer for a suitably matched donor cornea.

Lens Opacities Case-Control Study

Cataracts are a leading cause of visual disability and blindness, however, little information exists on the cause or progression of cataracts. Development of each cataract type (nuclear, cortical, mixed, and posterior subcapsular) could be influenced by different risk factors. The Lens Opacities Case-Control Study (LOCS) evaluated medical, nutritional, demographic, familial, environmental, and ocular factors that could lead to cataract development. Of the 1,380 LOCS study participants, 435 were cataract free. Cases of cataract were as follows: 72 with posterior subcapsular cataract, 137 nuclear cataract, 290 cortical cataract, and 446 with mixed types of cataract. The results of the study indicate that development of all three cataract types was associated with a lower educational level; and regular use of a multivitamin dietary supplement decreased the risk of cataract formation. Low dietary intakes of vitamins A, C, and E, riboflavin, niacin, thiamin, and iron were associated with development of cortical and mixed cataracts (odds ratios .31 to .56). Low intake of vitamins, low socioeconomic status, diabetes, race, use of some medications, smoking, and other factors were associated with development of specific types of cataracts. The rate of cataract progression and factors affecting this progression are currently being evaluated in the Natural History of Lens Opacities Study, where individuals will be examined annually for five years.

Linxian Eye Study

The Linxian Cataract Studies sought to determine whether vitamin and mineral supplements were effective in preventing the development of lens opacities. In 1985, the NCI launched two nutrition intervention trials in Linxian, a county in north central China, whose population has chronic nutritional problems and high rates of esophageal and stomach cancer. Because the vitamins and minerals under study might have potential for preventing lens opacities, the NEI collaborated with the NCI to determine the effects of the supplements on the eye's lens. In 1991, the NEI provided support for eye examinations, including detailed lens evaluations, for participants in both trials.

In the first trial, participants were randomly assigned to either a multivitamin and/or mineral supplement or a placebo. Examinations were conducted on 2,141 participants who were between the ages of 45 and 74. The study found a 36 percent reduction in nuclear opacities among the oldest participants (ages 65 to 74) who took a multivitamin and/or mineral supplement.

In the second trial, participants were assigned randomly to various combinations of vitamins and minerals—a study design that allowed researchers to determine the effects of individual nutrients. Examinations were conducted on 3,249 participants who were also between the ages of 45 to 74. The results indicated a significantly lower prevalence of nuclear opacities in people taking riboflavin and niacin compared with those not taking these vitamins. Again, the oldest participants (ages 65 to 74) showed the greatest reduction, 44 percent, in nuclear cataracts.

Optic Neuritis

Optic neuritis is an acute debilitating inflammation of the optic nerve that affects more than 25,000 Americans each year, primarily women between the ages of 18 and 45. People with the disease usually have rapid vision loss and ocular pain. The ONTT compared oral corticosteroid, intravenous steroid followed by oral corticosteroid, and placebo for the treatment of new cases of optic neuritis. ONTT results showed that oral corticosteroid, the most common treatment for the disease, when used alone is ineffective in treating the disease and actually increases a person's risk for future attacks.

Strabismus

Strabismus can result in amblyopia, which is a major cause of vision loss in the United States. The causes of strabismus are not well understood, but a defect in central nervous system control over the oculomotor system is thought to play a role. Maternal cigarette smoking during pregnancy as a risk factor for childhood strabismus was recently evaluated. A population-based, case-control study was conducted and evaluated all incident cases of strabismus diagnosed during a 21-month period from 1985 to 1986 in nine pediatric ophthalmology centers in Baltimore. Cigarette smoking was associated with esotropia but not exotropia for those women who smoked throughout pregnancy. The association between maternal smoking and esotropia was only seen in low-birth weight infants and infants in the upper-half of the birth weight distribution. The

authors conclude that cigarette smoking may have a direct toxic effect on the developing nervous system, which can lead to abnormalities such as strabismus.

Division of Biometry and Epidemiology

Report of the Acting Director, Division of Biometry and Epidemiology

Roy C. Milton, Ph.D.

The Division of Biometry and Epidemiology (DBE) comprises a Clinical Trials Branch, an Epidemiology Branch, and a Biometry Section. Dr. Roy Milton is the acting director for the Division. Drs. Frederick Ferris III and Robert Sperduto serve as chiefs of the two Branches, respectively; Dr. Roy Milton is the head of the Biometry Section.

The DBE has three main functions: research, education, and consultation. Research is the dominant function. It is the Division's mission to plan, develop, and conduct human population studies concerned with the cause, prevention, and treatment of eye disease and vision disorders, with emphasis on the major causes of blindness. This includes studies of incidence and prevalence in defined populations, prospective and retrospective studies of risk factors, natural history studies, clinical trials, genetic studies, and studies to evaluate diagnostic procedures.

The DBE carries out a program of education in biometric and epidemiologic principles and methods for the vision research community. This program consists of courses, workshops, a fellowship program for ophthalmologists, publications, and consultation and collaboration on research.

The Division provides biometric and epidemiologic assistance to NEI intramural and extramural staffs and to vision researchers in the public and private sectors. The assistance ranges from consultation to collaboration as coinvestigator.

Research Highlights

The Krypton-Argon Regression Neovascularization Study

This randomized multicenter clinical trial was designed to compare the efficacy of red krypton with blue-green argon laser photocoagulation for the management of high-risk proliferative diabetic retinopathy. Scatter laser photocoagulation with either argon or krypton appears to be equally effective in arresting neovascularization of the disc.

The Linxian Cataract Studies

The Linxian Cataract Studies were two randomized clinical trials conducted in China that studied the effect of vitamin and/or mineral supplements on the risk of developing age-related cataracts. In these studies of populations with chronic deficiencies of multiple nutrients, use of the supplements was associated with a decreased risk of nuclear cataract. Additional research is underway to determine whether these findings apply to less nutritionally deficient populations.

The Eye Disease Case-Control Study

In a large epidemiologic study of neovascular AMD, an increased risk of disease was associated with cigarette smoking and higher levels of serum cholesterol. Decreased risk was associated with postmenopausal use of estrogens and higher serum levels of carotenoids. Results from the study are consistent with a hypothesis linking risk factors for cardiovascular disease with AMD.

The hypothesis that higher serum levels of micronutrients with antioxidant capabilities may be associated with a decreased risk of AMD was evaluated in the Eye Disease Case-Control Study. Persons with higher levels of carotenoids and those with higher levels of an antioxidant index derived from serum measurements of vitamins C and E, carotenoids, and selenium also showed a decreased risk of macular degeneration. Results from this observational study are now being tested in a clinical trial.

A study was conducted to evaluate the relative anatomic position of the crossing vessels at the site of occlusion in eyes with branch retinal vein occlusion. In 99% of eyes with a branch retinal vein occlusion, the artery was located anterior to the vein at the obstructed site. At comparable nonoccluded crossings, the artery was located anterior to the vein less than 65% of the time. The finding suggests a possible role for mechanical obstruction in the pathogenesis of branch retinal vein occlusion.

In a study designed to identify risk factors for idiopathic rhegmatogenous retinal detachment, only one clearly relevant risk factor, myopia, emerged from the analysis. An eye with a spherical equivalent refractive error of -1 to -3 diopters had a fourfold increased risk of retinal detachment compared with a nonmyopic eye. Data from the study suggest that almost 55% of nontraumatic detachments in eyes without previous surgery are attributable to myopia. Results from the study are consistent with a hypothesis suggesting that the etiology of retinal detachment is related to the architecture of the eye, rather than to systemic factors.

A large epidemiologic study reported an increased risk of branch retinal vein occlusion in persons with a history of systemic hypertension, a history of cardiovascular disease, an increased body mass index at age 20, and a history of glaucoma. Risk of vein occlusion decreased with higher levels of alcohol consumption and high-density lipoprotein cholesterol. The data suggest a cardiovascular risk profile for patients with branch retinal vein occlusion and indicate that 50% of branch retinal vein occlusions may be due to hypertension.

The Sorbinil Retinopathy Trial

This multicenter trial was designed to assess the ability of sorbinil, an aldose reductase inhibitor, to retard the development and progression of diabetic complications. Results for retinopathy have previously been published. The study now reports that no benefit was found from sorbinil in slowing the development of clinical diabetic polyneuropathy.

The Early Treatment Diabetic Retinopathy Study

This multicenter, randomized clinical trial of aspirin versus placebo among diabetics examined mortality and morbidity from all causes with special emphasis on cardiovascular events. There were no harmful effects of aspirin, and the suggestion of beneficial effects was similar to previous studies of mainly nondiabetic persons.

Research Activities

Clinical Trials

The Early Treatment in Diabetic Retinopathy Study

The Early Treatment in Diabetic Retinopathy Study (ETDRS) was designed to determine when to use photocoagulation for diabetic retinopathy. Patients with macular edema, preproliferative retinopathy, and mild or moderate proliferative retinopathy were studied. Three forms of photocoagulation treatment, ranging from restricted focal treatment to complete panretinal photocoagulation, were compared with no photocoagulation. In addition, the study evaluated the placebo-controlled effects of daily administration of aspirin on the incidence of microvascular and macrovascular complications. The study also investigated factors associated with the progression of disease.

Recruitment was completed in March 1985 with the enrollment of 3,711 patients. In December 1985, the study reported that focal photocoagulation of clinically significant diabetic macular edema substantially reduces the risk of visual loss. It was further reported that focal treatment increases the chances of visual improvement, decreases the frequency of persistent macular edema, and causes only minor visual field losses.

Sixteen ETDRS reports have been published. Additional manuscripts are in preparation. Drs. Lloyd Aiello and Frederick L. Ferris, III serve as cochairmen, Dr. Richard L. Mowery is project officer, and Dr. Emily Y. Chew serves as a member of the analysis planning group. The ETDRS results of aspirin effects on mortality and morbidity in patients with diabetes were analyzed and published. Analyses in progress include the effect of aspirin on vitreous hemorrhage, risk factors for severe visual loss, and risk factors for development of high-risk proliferative diabetic retinopathy. In addition, patients with mild to proliferative retinopathy are

being followed with extensive psychophysical testing in the NEI Clinical Center to determine the mechanisms for loss of visual acuity in diabetic retinopathy.

The Sorbinil Retinopathy Trial

Dr. Daniel Seigel served as project officer for the Sorbinil Retinopathy Trial (SRT) until his retirement in November 1991. Sorbinil, a drug manufactured by Pfizer Laboratories, is an aldose reductase inhibitor that has potential to prevent or retard diabetic neuropathy and retinopathy. The NEI provided scientific leadership for this multicenter clinical trial, which was funded by Pfizer. Approximately 500 patients were randomized to treatment and followup, which ended in mid-1988. The results for retinopathy were published in 1990. No large benefit of treatment was observed. The effect of the treatment on neuropathy was summarized in a paper that was published this year.

The Krypton-Argon Regression of Neovascularization Study

The Clinical Trials Branch began the Krypton-Argon Regression of Neovascularization Study (KARNS) in three pilot clinics in December 1983. The major objective of this randomized clinical trial is to compare krypton laser with argon laser panretinal photocoagulation for treating neovascularization on the optic nerve head caused by diabetic retinopathy. Twenty-nine new clinics were enrolled in KARNS starting in August 1984. At the termination of the study in June 1990, a total of 1,063 patients had been randomized. This study is unique for the NEI because the functions for both the coordinating center and the fundus photography reading center are being handled by staff of the Clinical Trials Branch. Another feature of this multicenter trial is that the participating clinics receive no financial reimbursement from the NEI for their participation. Drs. Ferris and Chew direct this study along with Dr. Lawrence Singerman. Results of the KARNS were presented at the American Academy of Ophthalmology (AAO) in November 1992 and will appear in *Ophthalmology*.

The Linxian Eye Study

The NEI joined an ongoing NCI-supported clinical trial of nutrition and cancer in north central China in 1991 to determine whether the vitamin and/or mineral dietary supplements administered in the Linxian Cancer Trials for the preceding five years have affected the risk of age-related cataract and AMD. Eye examinations were conducted in 1991 on 5,390 members of the Linxian Study cohort. Dr. Sperduto is project officer, and the project team includes Drs. Milton and Chew from DBE and a Chinese ophthalmologist, Dr. Tian-Sheng Hu, from Beijing. Findings for cataract were published this year and are discussed in the research results section of the report of the Division of Collaborative Clinical Research.

Intramural Program Clinical Trials

Drs. Ferris and Chew are collaborating with Dr. Robert B. Nussenblatt on four additional randomized clinical trials in the NEI Intramural Program of the Clinical Center: (1) a trial of a sustained-release intraocular drug delivery system for gancyclovir therapy of CMV in patients with AIDS; (2) a trial to evaluate the efficacy of a heparin-surface modified intraocular lens in reducing the incidence and severity of postoperative inflammatory episodes following extracapsular surgery in uveitis patients with cataracts; (3) a trial of anti-inflammin, a peptide, in the treatment of anterior uveitis; and (4) a trial of S-antigen tablets in patients with uveitis.

Other

Dr. Seigel, as a special expert, continues to represent the NEI on the Data Monitoring Committee of the United Kingdom Prospective Diabetes Study, a clinical trial of alternative treatment regimens in the management of patients with diabetes. Followup is scheduled to continue in this study until 1994.

Epidemiology

The Age-Related Eye Disease Study

The Age-Related Eye Disease Study (AREDS) is designed to collect natural history data of 4,600 patients between the ages of 55 and 78 years with bilateral drusen of different types or with unilateral advanced AMD. This study will evaluate the rates of development and progression of AMD, the rates of visual loss due to retinal lesions of AMD, and the risk factors associated with the development and progression of AMD. Evaluation of lens change during the 10-year AREDS study period will provide an opportunity to evaluate factors associated with the

development of cataracts. In addition, a clinical trial will be performed to determine whether antioxidants (vitamins C, E, and beta-carotene) and zinc would prevent the development or retard the progression of AMD and cataract. There are 11 Clinical Centers, a Photographic Reading Center, a Central Laboratory, and a Coordinating Center. Identification of study participants began in September 1990. In November 1992, participants were evaluated with qualifying visits, and participants were randomly assigned to the study medications beginning in February 1993. Drs. Ferris (chairman), Sperduto (director of Lens Project), and Chew are directing the scientific aspects of the AREDS; Dr. Natalie Kurinij is the project officer.

The Eye Disease Case-Control Study

The Eye Disease Case-Control Study (EDCCS) is designed to identify risk factors for neovascular macular degeneration, idiopathic branch retinal vein occlusion, idiopathic central vein occlusion, rhegmatogenous retinal detachment, and idiopathic macular hole. Dr. Sperduto is study chairman, Ms. Rita Hiller is director of Data Analysis, and Dr. Chew is a member of the project team. All data have been collected. Five manuscripts were published this year: *Risk Factors for Neovascular AMD, Antioxidant Status and Neovascular AMD, Arteriovenous Crossing Patterns in Branch Retinal Vein Occlusion, Risk Factors for Idiopathic Rhegmatogenous Retinal Detachment,* and *Risk Factors for Branch Retinal Vein Occlusion.*

The Diabetes in Early Pregnancy Study

Dr. Emily Chew and Ms. Nancy Remaley, in collaboration with Dr. James Mills of the National Institute of Child Health and Human Development (NICHD), are examining the effects of pregnancy on diabetic retinopathy in the Diabetes in Early Pregnancy Study (DIEPS). Data collection terminated in 1985. A manuscript has been prepared.

The Italian-American Natural History Study of Age-Related Cataract

In Parma, Italy, the Italian-American Natural History Study of Age-Related Cataract will estimate the rates of development and progression of the different types of lens opacities and the associated risk factors. Dr. Sperduto is the project officer; Dr. Milton and Ms. Remaley are on the project team. Data collection began in May 1989 with baseline data from the Italian-American Case-Control Study of Senile Cataract and was completed in May 1993. Analyses of development and progression of age-related cataract are under way.

The Framingham Offspring Eye Study

Dr. Sperduto is the project officer, Dr. Milton is the alternate project officer, and Drs. Marvin J. Podgor and Valeria Freidlin and Ms. Hiller are members of the project team for the Framingham Offspring Eye Study (FOES). This study is designed to examine familial relationships for age-related cataract and AMD among parents examined in the Framingham Eye Study (1973-1975) and their children examined between 1989 and 1991. Dr. Podgor has used generalized estimating equation methodology in the analyses of these data. A manuscript describing the study's findings for cataract has been prepared.

Other

A manuscript has been submitted for publication on risk factors for strabismus, using data from the NICHD Collaborative Study and in collaboration with Dr. Mark Klebanoff, NICHD. The DBE project team includes Drs. Chew, Tamboli, Zhao, Podgor, and Ms. Remaley.

Statistical Methods

Dr. Marvin Podgor and Dr. Joseph Gastwirth from the George Washington University collaborated in the investigation of various tests for the two-sample problem with location and scale change alternatives. Dr. Podgor presented some of these results at the NIH Conference on Current Topics in Biostatistics and at the 1993 Joint Statistical Meetings. A paper has been accepted for publication.

Professional Activities

Members of DBE are active in consultations and educational and professional activities, including referees for professional journals, associate editors or members of editorial boards, members of data and safety monitoring committees for clinical trials, training of staff fellows, invited and

contributed presentations at professional society and other meetings, advisory committees for grant-supported cooperative agreements, and technical advisors to the World Health Organization (WHO).

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International Program Activities

Report of the Acting Assistant Director for International Program Activities

Terrence Gillen, M.A., M.B.A.

The mission of the NEI includes the reduction of the prevalence of blindness, visual impairment, and eye disease worldwide through basic and applied research and training. Although excellent ophthalmic procedures and eye-care delivery systems are accessible in the developed world, adequate health care is not readily available in all parts of the developing world. This widening gap in visual health between developed and developing nations threatens to have ominous consequences. If present trends continue, the number of blind people—today estimated at 24 million—will more than quadruple during the next 40 years. Tragically, as many as 90% of these blind people will live in developing countries.

This large-scale disablement caused by blindness is not only a costly obstacle to economic development, it is a catastrophic loss of human potential in the very parts of the world most desperately in need of a healthy workforce. In addition, because more than 80% of all cases of blindness can be considered avoidable—that is, they could have been prevented or could be cured using available and locally appropriate technology—such deprivation is a truly needless denial of a basic human right for millions and millions of people. Therefore, the NEI undertakes international activities to facilitate the development and application of effective prevention and intervention programs. These efforts are coordinated by the Institute's Office of International Program Activities (OIPA), which was created in February 1989. OIPA enhances NEI's international programs, which include:

- Evaluating available health technologies, promoting the most cost-effective intervention and prevention programs, and encouraging their availability for affected populations, especially in developing countries.
- Conducting collaborative applied research studies to develop preventive methods for treating specific eye diseases.
- Conducting controlled clinical evaluations of promising research findings.
- Exchanging information on recent scientific advances and their appropriate application to visual problems.

NEI currently supports international research on several blinding diseases that have a major worldwide impact: cataract, onchocerciasis, ocular toxoplasmosis, glaucoma, diabetic retinopathy, and vitamin A deficiency. During the past year, the NEI has continued to support investigations of important blinding eye diseases with worldwide impact. These studies are implemented through bilateral agreements with the U.S. Government, other types of country-to-country programs (such as those supported by the U.S. Agency for International Development [U.S. AID]), and through collaborative activities with the WHO, the Pan-American Health Organization, and with foundations and private and voluntary organizations such as the International Association of Lions Clubs.

Research Activities

Cataract

Because cataract is responsible for about one-half of the developing world's curable blindness and is a major problem for the United States as well, the NEI has developed a collaborative research program that includes projects to prevent blindness from cataract with collaborating groups in Italy, India, and Latin America. Additionally, health services research expertise from the NEI is made available to selected collaborating partners through training activities and the conduct of joint research projects.

An NEI-supported randomized clinical trial to compare intracapsular cataract surgery plus aphakic spectacles with extracapsular cataract extraction plus implantation of an intraocular lens (IOL) is being conducted at the Aravind Eye Hospital in Madurai, India. The trial's primary comparison concerns operative and postoperative complications.

Secondary evaluation endpoints include measurement of vision function assessed by interview using a multi-item questionnaire and appraisal of economic impact in terms of direct and indirect cost associated with blindness and cataract surgery.

The Collaborative Italian-American Case Control Study of Age-Related Cataract, which was started in September 1986, as part of the program of cooperation in biomedical research between the United States and Italy, has now been completed. The objectives of the study were to: (1) identify risk factors for age-related cataract, (2) evaluate methods of *in vivo* cataract classification, and (3) compare the findings with those of parallel studies being conducted in the United States and India. Data were collected at the Institute of Ophthalmology at the University of Parma. The Laboratory for Epidemiology and Biostatistics at the Istituto Superiore di Sanita in Rome served as the study's coordinating center. Data collection ended in April 1989, after 1,477 subjects had been entered into the system. Four papers describing the study's findings have been published.

The Collaborative Italian-American Study of the Natural History of Age-Related Cataract has added a four-year followup component to the recently completed case-control study of age-related cataract. Approximately 1,000 subjects with cataracts and 300 subjects free of cataracts are being examined every six months for four years to collect data on the natural history of the various types of cataracts. Data collection began in May 1989. The objectives of the natural history study are to estimate the rates of development and progression of the various types of lens opacities, identify risk factors associated with the development and progression of cataracts, and determine whether the risk factors affecting rates of progression differ for the various types of lens opacities.

Data collection ended in April 1993 at the Institute of Ophthalmology, University of Parma. The Laboratory for Epidemiology and Biostatistics, Istituto Superiore di Sanita in Rome serves as the Coordinating Center. Preliminary findings from the study were presented in Stresa, Italy, at the 1992 International Congress of Eye Research meeting. Preliminary findings from the study were presented at the meeting of the Association for Research in Vision and Ophthalmology in May 1993. A paper describing incidence and progression rates for specific cataract types has been prepared for publication.

International collaborators have been established by scientists in the NEI's Laboratory of Mechanisms of Ocular Diseases, Section on Cataract to further our understanding of the relationship between enzyme deficiency diseases and cataract. For example, these NEI scientists have begun a candidate gene study to determine whether a deficiency in sorbitol dehydrogenase (SDH) in a family where several members have congenital cataracts is due to changes in SDH gene structure or expression. This study is possible through the cooperation of the Unidad de Investigacion Biomedica Hospital de Pediatria, Instituto Mexicano del Soguro Social, Guadalajara, Mexico.

Vitamin A Deficiency

Although not a major problem in the United States, vitamin A deficiency worldwide affects an estimated 14 million children annually. It is the world's major cause of childhood blindness, accounting for 250,000 to 500,000 new cases of blindness per year. In addition, children die at higher rates from common childhood infections if they are deficient at any level of severity. The NEI supports basic research on the interaction of nutrients such as vitamins A, C, and E on retinal and other eye tissue development. Such investigations can lead to clinical interventions that may help alleviate morbidity from malnutrition eye disease. In addition, the NEI has provided technical consultation for a study in south India that has shown an impressive reduction in childhood mortality associated with improved vitamin A nutritional status, and other efforts to transfer this technology to alleviate world blindness are under way.

Particularly in Asia, vitamin A deficiency is a public health problem and the leading cause of blindness among preschool-age children. The most effective way of providing affordable prevention programs is under study by the University of Michigan and the Nepal Netra Jyoti Sangh, Nepal's national society for the prevention and control of blindness. OIPA is providing technical oversight for this three-year project for the U.S. Department of Health and Human Services (DHHS) Office of International Health and the U.S.AID East Bureau.

Glaucoma

Open-angle glaucoma is the leading cause of blindness among African Americans and is a major cause of visual impairment and disability. The incidence of glaucoma has not been measured precisely in any population, and the risk factors related to its development are largely unknown. In the Barbados Eye Study more than 4,200 persons between the ages 40 and 86 years were examined from 1988 to 1992 as part of a population-based study to determine the prevalence and risk factors for glaucoma and other eye disorders such as diabetic retinopathy, AMD, cataract, and visual impairment. In 1992, the Barbados Incidence Study was initiated to estimate the incidence of glaucoma and other ocular disorders from individuals free of disease in the Barbados prevalence survey. Also, risk factor analysis will be conducted for associations with development of glaucoma and to characterize those who have progressive eye disease.

The Early Manifest Glaucoma Trial is a randomized, controlled clinical trial designed to determine whether and to what extent reduction of IOP influences the course of chronic open-angle glaucoma. Investigators at the University of Lund in Malmo, Sweden, collaborating with investigators at the State University of New York at Stony Brook, will study an estimated 300 patients with newly diagnosed disease. Participants will be randomized either to pressure-lowering treatment or to observation without treatment. Both groups will be followed closely with computerized perimetry and fundus photography. Recruitment of patients began in 1993 and will continue for an estimated two years. Followup of patients will be conducted for four years.

Retinal Degenerations

In collaboration with protein biochemists at the Karolinska Institute in Stockholm, Sweden, NEI cataract researchers are investigating the evolutionary relationships of ζ-crystallin, an enzyme/crystallin of certain species, with other oxido-reductases. Establishing such relationships with enzymes of known function should help in identifying the physiological roles of ζ-crystallin both in the lens and in other tissues where it is present at low levels.

Diabetic Retinopathy

The United Kingdom Prospective Diabetes Study is a prospective randomized study of different therapies to determine whether improved blood glucose control or improved blood pressure control of noninsulin-dependent diabetes will reduce morbidity and mortality. The study began in 1977 and has recruited 5,102 newly diagnosed diabetic patients. Patients who fail to respond to diet therapy are randomized to diet therapy or "active therapy" with sulfonylurea, insulin, or metformin. As part of the study, hypertensive diabetic patients have been randomized to "tight blood pressure" control with either an ACE inhibitor or beta-blocker to "less tight control." The development and progression of diabetic retinopathy in these patients is being assessed by retinal photography. The study is currently completing 10 years of patient followup.

Management for Eye-Care Delivery Course

As a WHO Collaborating Center for the Prevention of Blindness, the NEI offered a course at the Aravind Eye Hospital in Madurai, India, in January 1993, on management for eye-care delivery.

The purpose of this five-day course was to enhance the effectiveness of mid- and senior-level managers of eye-care programs and facilities. The course broadened the management perspective of the students, extended their understanding of decision-making, and developed their problem-solving skills. Emphasis was placed on demonstrating the application of operations research and management science to "real world" problems.

Individually and in small discussion groups, participants analyzed each case by identifying the basic problems involved, characterizing the relevant background setting and facts, formulating an appropriate analytical framework or model, and generating alternative solutions. Subsequently, in a large-group classroom setting, all participants exchanged views concerning the cases and tested their conclusions. Group discussion forced participants to examine critically their own assumptions and to narrow their thinking to a plan of action. Members of the faculty guided the classroom discussion and ensured that all significant issues were addressed and that there was full participation.

Consultation to the World Bank

The director, deputy director, and special advisor to the director, NEI, have participated as consultants to the World Bank in the development of a proposal by the Government of India for a major initiative in cataract blindness control. Technical meetings were held in New Delhi and Madurai to provide the knowledge base upon which training and surgical guidelines can be developed for a significant expansion of cataract surgery with explicit attention to the quality and extent of vision restoration.

Activities With International and Multinational Organizations

In FY 1993 NEI staff continued to provide technical advice to Lions Clubs International in the development of their $100 million SightFirst initiative, a global sight-conservation program aimed at substantially reducing the prevalence and incidence of preventable and curable vision loss.

Also in FY 1993, NEI continued its activities as a WHO Collaborating Center for the Prevention of Blindness. The NEI director continues to serve on the WHO's Special Advisory Panel in the Prevention of Blindness, and the assistant director for International Program Activities serves on the Global Advisory Committee. Other NEI staff members have, on request, given consultations to the WHO program. In addition, an ophthalmologist from India visited the NEI, under the auspices of a WHO fellowship, to study cataract etiology and prevention.

NEI continues working closely with nongovernmental organizations in designing service and research programs to reduce the prevalence of blindness, regardless of its etiology, throughout the world.

Extramural Programs

In FY 1993, NEI granted 14 awards to foreign institutions in eight countries. Research and training projects were supported in lens and cataract, glaucoma, visual system development, photoreceptors, phototransduction, visual cortex, visual abnormalities, Leber disease, nutrition of the eye, ocular complications of diabetes, and the prevention of blindness. Awards covered both basic and clinical research projects.

Intramural Programs

NEI continues to serve as an international center for research and training on eye disease. In FY 1993, 18 visiting fellows, 22 visiting associates, 13 visiting scientists, 21 special volunteers, and eight guest researchers, from more than 20 countries conducted research in NEI's Bethesda, Maryland, facilities. Their work included basic laboratory investigations on the molecular structure and development of the visual system, sensory and motor disorders of vision, and the biochemical bases of retinal and corneal diseases and cataract development. In addition, visiting scientists collaborated with NEI investigators in clinical studies to define, treat, and prevent vision disorders such as genetic and developmental defects, ocular inflammatory disease, and ocular complications due to systemic conditions such as diabetes.

Science Policy and Legislation

Report of the Associate Director for Science Policy and Legislation

Michael P. Davis, M.S.

The Office of Science Policy and Legislation (OSPL) is responsible for a broad and diverse range of management activities that support and further the NEI's mission. Among these are providing leadership and direction for program planning, analysis, evaluation, and legislative functions, including the development and maintenance of a computerized management information system and public information and scientific reporting services in support of the NEI's research programs.

This year has been a period of significant change and yet has also been a time of significant accomplishment. Mr. Julian Morris, who had served the NEI for more than 20 years in positions ranging from information officer to associate director for science policy and legislation, succumbed after a lengthy illness. As a tribute to his numerous contributions to the institute, the NEI staff and the National Advisory Eye Council (NAEC) honored his memory by dedicating the most recent long-range plan for vision research to him.

During this past fiscal year, the three sections that make up the OSPL were elevated to branch level. Mr. Michael P. Davis, who had served as acting associate director during Mr. Morris' illness, was selected to fill the vacancy. Subsequently, Dr. Carmen P. Moten, a program analyst within the Policy, Legislation, Planning, and Evaluation Branch (PLPEB), was selected as chief of that branch.

Also of great significance was the completion of *Vision Research—A National Plan: 1994-1998.* After final updating by staff, the plan was sent to MERIT awardees for their comments and suggestions, prior to final review and approval by the NAEC. Development and publication of such a comprehensive plan by so small an office, especially during a period complicated by many external factors, was an enormous undertaking. Without the hard work and extra effort by Dr. Moten and Mr. Whitaker of the PLPEB, Dr. McLaughlin and the program directors from the extramural research program, and a very talented group of publication experts at CSR, Inc., final publication would not have occurred. We are greatly indebted to them for their assistance in bringing this excellent document to completion.

Policy, Legislation, Planning, and Evaluation Branch

Carmen P. Moten, Ph.D., Chief

The PLPEB advises the NEI director on program planning, analysis, evaluation, and legislation and serves as the focal point within the Institute for these functions. In addition, PLPEB develops and executes a comprehensive program planning strategy for the Institute, including the periodic development of a national five-year vision research plan in conjunction with the NAEC; plans, coordinates, carries out, and/or monitors NEI program evaluations; and prepares recurring and ad hoc program analyses in response to requests from the NIH, the Public Health Service (PHS), and the Department.

During FY 1993, the principal activities for the PLPEB were:

- Preparation of material for the 1993-1994 *Biennial Report of the Director, NIH*, describing research accomplishments, outlining future opportunities, and assessing important policy issues.
- Preparation of briefing materials for the confirmation hearing of NIH Director-Designate Dr. Harold Varmus.
- Support of the NEI extramural grant information retrieval system by assigning scientific, biotechnology, diabetes, and other codes to awarded grants for the purpose of analyzing and reporting research activity in areas of interest to the NEI, NIH, DHHS, Congress, or non-governmental organizations and individuals.

- Preparation of materials for the Congressional Appropriations Committee Report—*Decade of the Brain.*
- Preparation of materials for NIH-coordinated activities with PHS agencies.
- Preparation for publication by NIH *The 1993-1994 Prevention Annual Report.*
- Preparation of materials for the NIH director on supported research related to FDA-approved drugs.
- Preparation for publication by NIH of *The 1992 Annual Report on Rare Disease Research Activities.*
- Preparation of briefing materials for the General Accounting Office (GAO) on activities duplicated among PHS agencies.

The PLPEB has also been involved in researching, writing, and editing a variety of reports requested by the NIH, PHS, DHHS, Congress, and non-governmental organizations and individuals that include the following:

- Report to Congress on sickle cell anemia research and the role of minority institutions in research.
- Review of the draft update of the *Healthy People 2000: Public Health Service Action Report.*
- NEI submission on fibromyalgia for the *Congressional Appropriations Report.*
- NEI submission for the *Diabetes Mellitus Interagency Coordinating Committee (DMICC) Annual Report.*
- Scientific advances for the NIH FY 1994 Congressional Justification.
- Report on breast cancer-related research during FY 1992, in response to an NCI request.
- Recommendations for the implementation of the NIH Strategic Plan, in response to an OSPL request.
- Review of the draft report—*Public Version of the Strategic Plan.*
- Support of the NIH Conference on Disease Prevention Research.
- Review of the *Cross-Cutting for Healthy People 2000 Progress Report—American Indians and Alaskan Natives; Adolescents and Young Adults: Hispanic Americans; and Women.*
- Recommendations for technical changes to the H.R.4, the NIH Revitalization Act of 1993.
- Review of the draft report—*Clinician's Handbook of Preventive Services 1993* and *Clinical Preventive Services: What Works and What It Costs,* supported by the National Coordinating Committee on Clinical Preventive Services (NCCCPS).
- Report on space medicine-related research, in response to a NIH director request.
- Report on research use of FDA-approved drugs, in response to a Congressional request.
- Report on federally funded current research, training, and intervention projects related to injury control.
- Briefings for the GAO.
  - — Breast cancer
  - — Immunology
  - — Diabetes
  - — Environmental health
- Policy briefing materials for the director, NIH.
- Report on adolescent-related research, in response to a NICHD Office of Demographic and Behavioral Sciences request.
- Report on trauma-related research, in response to an NIH director request.
- Submission of highlights of drug-related research for Congressman Waxman's request for information concerning NIH-supported research on drugs.
- Final review of the draft report—*Implementation Plan on Health and Behavior Research.*
- Report to the Office of the Inspector General for an on site discussion of the Institute's mission and program activities.
- For the NEI Scientific Reporting Branch, data on intramural and extramural research in the following areas:
  - — Dry eye
  - — AMD
  - — Glaucoma
  - — Cataract
  - — RP
  - — Sjogren syndrome

  - — Ocular implants
  - — Corneas
  - — Retinal transplants
- For the NEI Financial Management Branch, information on intramural and extramural research costs for the following areas:
  - — Biotechnology
  - — Prevention
  - — Immunology
  - — Vaccine development
  - — *Decade of the Brain*
  - — Breast cancer
  - — Tuberculosis
  - — Aging
  - — Nutrition
  - — AIDS
  - — Women's health issues
  - — Diabetes
  - — Cancer
  - — Sexually transmitted diseases

The PLPEB also provided editorial review of a variety of letters, reports, and other narrative materials for other offices within the NEI.

Management Information Systems Branch

David Scheim, Ph.D., Chief

During the past fiscal year, the Management Information Systems Branch (MISB) has made significant changes to its local area network (LAN) configuration prompted by the move of the NEI's Extramural and Collaborative Program to Executive Plaza South (EPS) in April 1993. To accommodate this move, MISB configured a new LAN at EPS, connected to the NEI's Building 31 LAN via NIHnet using TCP/IP. MISB designed the star topology for the EPS LAN, based upon unshielded twisted pair UTP cabling.

To support this distributed configuration of two LANs connected over NIHnet, the total number of network file, print, and database servers was increased from six to eight. The MISB procured the needed computer hardware, designed the network cabling and EPS machine room arrangement, and reassembled all 40 workstations at EPS after the move. During the fiscal year, MISB also configured additional Dell 486 computers to replace the last of the NEI's outmoded Zenith 286 PCs, yielding a total of 95 NEI workstations. MISB also upgraded the network cards in all workstations to Etherlink II or III for improved performance and reliability.

During the past fiscal year, the MISB also upgraded all client network software to LAN Manager 2.1a. The network's database server was upgraded to a Dell 486/50 with 2.1 gigabytes of disk space to support greatly expanded database functionality. SQL Bridge was installed and configured to allow transparent access by Building 31 NEI staff to database systems, which used the NEI's SQL server located at EPS.

Remote printing capability via BARRHASP was added to the EPS LAN and was enhanced to allow more flexible routing to different network printers at the Building 31 site. Daily network backup procedures using two digital/audiotape (DAT) drives were enhanced to ensure more reliable operation. An uninterrupted power supply unit (UPS) was procured and installed at the NEI's EPS site, providing continued operation in the event of a temporary power loss and an automatic smooth network shutdown in the event of a protracted power outage.

The MISB provided extensive enhancements to its grants information systems during FY 93. Microsoft SQL Server, the database server for all systems, was upgraded to version 4.2 to allow enhanced functionality. JAM and JAM/DBI, the client tools for these systems, were also upgraded to allow additional functionality. The existing NEI snapshot, council letter, and grants coding systems were upgraded for this new environment.

Three new systems for, respectively, user-friendly grants queries, CRISP queries, and pay plan management were designed and programmed by MISB staff. Automated security and login functions integrated with LAN login were developed by MISB staff. These functions provide automatic login to grants information systems for NEI staff and also

automatic tracking of system usage. Database system status information has been integrated into this automatic logon procedure, so that users receive messages concerning system shutdowns when they occur and estimated times of resumed operation upon logon to these systems.

Automatic daily check and backup procedures have been implemented through custom programming by MISB for all active NEI databases. These procedures are automatically run overnight from an NEI LAN server with results automatically recorded in a log table that can be instantly checked by MISB staff.

The NEI's weekly grants update batch procedures have been fine-tuned to provide virtually no weekday system downtime during FY 1993. These procedures continue to be run each Sunday by MISB staff to allow full system availability during the work week.

During the end of the fiscal year, these update procedures, now running in Paradox, were completely reprogrammed by MISB staff in Microsoft SQL Server Transact SQL language. When tested and implemented in FY 1994, this reprogramming will allow Paradox operations to be fully superseded by the more reliable, state-of-the-art client-server architecture. This reprogramming effort will streamline the NEI's database configuration, provide enhanced reliability and maintainability, and establish a foundation for additional advanced system development for both grants and other NEI functions.

The MISB has continued to provide custom information reports to NEI staff for internal use and public distribution, with 107 new requests logged for FY 1993 and rapid turnaround achieved in every case. Weekly and monthly reports, as well, continue to be provided. In addition to its own programming efforts, the MISB has continued to support NEI staff in the use of information resources provided by the Division of Research Services (DRG), the National Library of Medicine (NLM), and other sources, including the DRG information system, CRISP, FOCUS, WYLBUR, MEDLINE, Grateful Med, Legislate, the electronic NIH library catalog, Gopher, and other specialized systems.

During the past fiscal year, MISB has implemented upgrades to several of the software packages it operates while continuing to provide support for its full LAN software array, including Harvard Graphics, Quattro, Paradox, Calendar, FTP PC/TCP, Microsoft Mail, Virus scanner, and other packages used for specialized functions. The MISB has continued to support all computer hardware in-house, with service calls made only to order parts not internally available or to handle unusual problems. This internal support has resulted in substantial savings to the NEI.

The MISB has arranged for the services of a high school student intern during FY 1993, who assisted with its evaluation of a client-server gateway that the Division of Cancer Research and Treatment (DCRT) is now in the process of procuring for NIH-wide use. This gateway will allow NEI transparent access into personnel and administrative database systems whose data is stored in the DB2 mainframe database system. The MISB has continued to be a leader in the development of client-server database systems at the NIH, and MISB staff members have demonstrated NEI systems at various intercampus forums.

The MISB has continued to handle a number of IRM functions for the NEI, including its environment and resources report, strategic plan, tactical plan, budget report, and security functions. The MISB staff members have continued to represent the NEI on a number of NIH-wide committees, including the Office Technical Coordinators and its network subcommittee, the ADP Extramural Programs Coordinating Committee and its steering committee, the Database Technology Task Force, the NIH lead users group, the Campus Users Research Exchange, and the Technical LAN Coordinators Committee.

Scientific Reporting Branch

Judith A. Stein, M.A., Chief

This year, the Scientific Reporting Branch (SRB) provided numerous reporting and public information activities in support of NEI programs. Specific activities included the development of responses to inquiries received from the general public, professionals, and the media; the development and dissemination of information and education materials; development of A Celebration of Vision Research, highlighting the NEI's 25th anniversary; planning, implementation, and coordination of the

National Eye Health Education Program (NEHEP); maintenance of an NEI exhibit program; and preparation of reports to the Congress. Specific accomplishments in these areas are outlined below.

- The SRB responded to approximately 17,000 written, telephone, and in-person inquiries from the general public, patients and their families, students, health professionals, the media, and other professionals. This figure represents almost a threefold increase in inquiries from FY 1992. In addition, staff members responded to 15 pieces of controlled correspondence. These correspondences included responses to congressional inquiries and Presidential greetings and proclamations. To support the public inquiry function of this office, the SRB staff developed and/or updated publications, including a new brochure for people at risk for AMD.
- A new edition of the book, *Clinical Trials Supported by the National Eye Institute* was produced. This book describes 16 nationwide extramural clinical trials supported by the NEI and, for the first time, five intramural studies conducted at the NIH. The book will be promoted to practitioners through exhibits at the upcoming meetings of the AAO and the American Academy of Optometry and through public service advertisements and announcements in various professional journals.
- The results of the Retinitis Pigmentosa Vitamin Study were announced in May. This included the writing and dissemination of a press release to the print and electronic media. A "Dear Colleague" letter was prepared and distributed to all members of the AAO and the American Optometric Association.
- To highlight the NEI's 25th anniversary, A Celebration of Vision Research was initiated. The objectives of this activity are to provide the American public with a report on its investment in vision research, highlighting the achievements and frontiers of publicly funded vision research; increasing awareness of the benefits derived from vision research; and stimulating interest in biomedical research. A traveling science museum was developed that will present the progress and future of vision research by highlighting the anatomy and physiology of the eye and visual system through the use of interactive modules; artifacts from the past such as eyeglasses, advertisements, equipments, etc; common eye diseases and disorders, focusing on both basic and clinical research; and predictions for the future of vision research. In addition, the development of a school program for grades four to eight was initiated.
- The NEHEP continued to expand its efforts to reach people at risk for glaucoma or diabetic eye disease. This included the distribution of the three NEHEP education kits to individuals responsible for educating people at risk. Since the kits became available in the spring of 1992, more than 37,350 kits have been distributed. In addition, initial plans were developed to expand the diabetic eye disease program to reach Native Americans and Hispanics. This included conducting literature searches, identifying organizations, and reviewing current materials designed for these audiences.

  The membership in the NEHEP Partnership grew from 43 to 50 organizations. A Partnership action plan was developed to monitor activities as well as to identify opportunities for future involvement in the NEHEP. Technical assistance was provided to many community-based organizations to increase their participation in the NEHEP, particularly through the involvement of local Partnership members.

  Plans were made to hold the Third National Eye Health Education Conference in December 1993 in Pennsylvania. The purpose of this conference will be to foster the development of local eye health education programs. The conference will provide the NEHEP Partnership with the opportunity to work together and to learn new skills that can be used to implement programs at the local level.
- SRB staff members represented the NEI at the meetings of several professional organizations, including the American Diabetes Association, the Association of State and Territorial Directors of Public Health Education, the American Association of Diabetes Educators, and the National Association of Area Agencies on Aging.

Office of the Scientific Director

Report of the Scientific Director

Robert B. Nussenblatt, M.D.

This past year has been one of change and achievement in the Intramural Research Program of the National Eye Institute (NEI). Work has progressed in both clinical and basic research spheres. There has continued to be a heavy emphasis in the area of AIDS (acquired immune deficiency syndrome), with an ongoing clinical trial to evaluate the effectiveness of a sustained-release device for ganciclovir placed into the eye for the treatment of cytomegalovirus (CMV) retinitis. A randomized masked study to immunomodulate uveitis has continued. This attempt at using oral tolerization is an attempt to down-regulate inflammatory disease in patients' eyes without the use of medications.

In the area of basic research, there have been developments in exciting new concepts about gene regulation, as well as continued advancement toward the ultimate goal of the use of gene therapy to treat eye diseases. Following are a few highlights of research achievements by the NEI intramural scientists during Fiscal Year 1993.

Laboratory of Mechanisms of Ocular Diseases (LMOD)

LMOD investigators have continued studies on a broad range of topics relating to the biology of various tissues, looking in depth at the molecular mechanisms responsible for certain ocular disorders. The major emphases of this group continue to be cataract and the ocular complications of diabetes. This year the group has shown, using site-directed mutagenesis, that the histidine at position 110 of the enzyme aldose reductase appears critical for catalytic activity. Studies have been instituted to evaluate a family with congenital cataracts whose members have a probable defect in the sorbitol dehydrogenase gene.

On the clinical level the group also has evaluated the protein composition of normal human lenses and cataracts. Dr. Fielding Hejtmancik and his colleagues have been studying the structure and function as well as the relationships of β-crystallins. At the same time, they are conducting gene mapping studies on a variety of genetic disorders that have ocular manifestations, including Usher's syndrome type I. These researchers have mapped two independent genes to chromosome 2.

Dr. W. Gerald Robison has continued to refine and better characterize the rat model for diabetic retinopathy. Rats with this disorder will develop striking angiopathy in the retina.

Laboratory of Sensorimotor Research (LSR)

This Laboratory of international standing has continued to concentrate its research efforts on the brain mechanisms underlying the visual sense and visually controlled movements. Skilled motor control is one of the tasks the LSR scientists have concentrated on, particularly in relation to the visual control of eye movements. They have integrated, in a superb fashion, the observations made in humans, using an excellent animal model, the Rhesus monkey. Its use permits exploration of not only the exact behavioral mechanisms related to visual motor behavior but also the underlying brain mechanisms. One area of particular interest has been the generation of rapid or saccadic eye movements.

Dr. Fred Miles and his group have attempted to increase understanding of the problem solving that goes into generating very rapid saccades. Some work has centered on the evaluation of what happens when monkeys or humans are confronted with different-sized images that are presented before each eye. Building on past observations, Dr. Miles' group has found that humans immediately adjust the amplitude of their eye movements in ways that are appropriate to the size of the stimulus. These LSR researchers have conjectured that the horizontal disparity in the images detected by the visual system controls this movement. They were able to reproduce this phenomenon in the monkey, thereby

permitting further evaluation of the brain mechanisms underlying this control. In parallel fashion, Dr. Wurtz and his group have evaluated the control of movement through the environment and the stabilization of posture.

Dr. Lance Optican, who has an ongoing interest in the way neurons convey visual information, has demonstrated that neurons convey information about visual features by using a temporal code. This work may elucidate the fascinating concepts of how the brain processes information that ultimately leads to visual perception.

Dr. Michael Goldberg and his collaborators have used a variety of techniques to evaluate the control of eye movements in the monkey. More recently they have applied these techniques to the human situation. In recent work, using PET scanning, they have identified in the human the approximate region in which frontal eye fields are located, which they had previously noted in the monkey.

During this past year the whole LSR moved to the new Silvio O. Conte Building. This facility provides a state-of-the-art environment for nonhuman primate research.

Laboratory of Ocular Therapeutics (LOT)

This Laboratory has continued to focus on the development of new ophthalmic drugs—aldose reductase inhibitors as well as anticataract agents. This year more effective and less toxic aldose reductase inhibitors that are unrelated to previous aldose reductase inhibitors have been developed through the use of biochemical, pharmacological, and computer molecular design techniques.

The Laboratory has continued long-range studies evaluating the effect of galactose on dogs. The retinal changes that have been seen are typical of diabetic retinopathy as it progresses to the proliferative stage. This dog model is the first experimental model that demonstrates both clinical and histological changes found in all stages of diabetic retinopathy.

Laboratory of Molecular and Developmental Biology (LMDB)

LMDB investigators continue to focus on understanding the fundamentals of gene expression and cellular differentiation in the eye. Major emphasis is placed on the lens. On the basis of their earlier observations that lens crystallins appear to be multifunctional proteins that are expressed outside the lens and eye, they have broadened their research during the past year to include new areas of metabolism and gene expression in various tissues. Of interest is the fact that transcriptional factors involved in the expression of crystallin genes are present in many tissues and are used to control numerous biological processes. The implications of gene control in the eye go far beyond this organ.

Of great importance this year has been the LMDB's identification of regulatory elements required for expression of genes in the eye and other tissues. Regulatory elements may be functionally redundant, that is, removing a regulatory element will not necessarily eliminate expression of the gene. A great deal of emphasis has been placed on the expression of proto-oncogenes and cyclins, which have been linked to the normal processes of cellular differentiation in the lens, as well as to the cell cycle and growth control.

The development of a transgenic facility within this Laboratory has expanded the NEI's ability to use transgenic animal models. An ongoing, fruitful interaction between the LMDB and the Laboratory of Immunology (LI) has resulted in genetic engineering experiments which have the potential to contribute animal models for research on autoimmune diseases of the eye.

Ophthalmic Genetics and Clinical Services Branch (OGCSB)

The OGCSB conducts clinical and laboratory research on gene expression and molecular interactions important to the eye. There is also an

application of clinically relevant research findings for the prevention, diagnosis, and treatment of diseases affecting the eye and visual system. The Branch has continued using different systems to develop objective and subjective methods of monitoring and documenting opacities in the human lens. Among various objective systems that have been utilized are the Scheimpflug camera and the retroillumination camera. Other subjective systems utilized include the LOCS-2 grading system, as well as contrast sensitivity and glare testing.

The OGCSB has continued its ongoing cataract research, carefully documenting cataracts in patients using a variety of techniques. Small pieces of tissue from cataracts extracted extracapsularly are used for various laboratory tests. Abnormal proteins have been identified by immunoblotting techniques as well as protein sequencing. It has been shown that in aging there is an acidic shift of proteins and that an increased number of polypeptide species exist within the molecular weight range of the crystallins.

The Branch also has been a leader in the study of gyrate atrophy. Dietary intervention studies will continue in families with two affected siblings. A marked decrease in the retinal progression of this disorder now can be seen in the children who began the dietary intervention at an early age. This original work will lead to the exciting area of gene therapy. These studies will be performed in collaboration with the Laboratory of Immunology (LI).

Laboratory of Retinal Cell and Molecular Biology (LRCMB)

The research focus of the LRCMB has been to elucidate new genes and biochemical mechanisms that deal in particular with the retinal pigment epithelial (RPE) complex, in both health and disease. The long-term goal of this group is to establish basic information that will permit the Intramural Program to apply rational methods of gene therapy to various disorders. Several retina-specific genes identified by subtractive cloning have been located on the short arm of the X chromosome.

In a similar fashion, LRCMB scientists have cloned RPE-specific genes that appear unique to the RPE. A new 65-kD protein of potential immunologic importance has been isolated from the human RPE and its gene has been cloned. This is the first RPE-specific gene to be reported and characterized.

Work also has centered around a pigment epithelium-derived factor, which in very low concentrations causes the extension of elaborate neuronal processes from cultured retinoblastoma cells. It is hoped that this work may ultimately lead to understanding cone neuron development.

LRCMB researchers continue to collaborate with LI investigators in studies of the immunopathology of experimental autoimmune uveitis.

Laboratory of Immunology (LI)

The LI is dedicated to the evaluation, diagnosis, and treatment of ocular inflammatory diseases. The research carried out by this group is both clinical and basic. In the clinical arena, the group continues to have a major commitment to the study of the ocular complications of AIDS. The group has been interested in establishing noninvasive methods for diagnosing the presence of CMV retinitis in a non-ophthalmic setting. The use of the cell flare meter has helped in this regard. It shows an increase in the protein content in the anterior chamber that appears in AIDS patients who have CMV retinitis. This machine also is being used for a variety of therapeutic studies.

A randomized study to evaluate the usefulness of an intraocular slow-release device containing ganciclovir that releases the drug for an 8-month period was in progress this past year. Patient recruitment continues but will end shortly. Newer medications such as rapamycin have been studied extensively in the experimental uveitis model. In addition, planning has begun for the use of monoclonal antibody therapy. The initial target is the interleukin 2 (IL-2) receptor.

The Laboratory has had a long-term interest in toxoplasmosis. During the past year LI researchers have developed a reliable ocular model for toxoplasmosis in which retinal as well as brain cysts of this origin can be demonstrated. This model has been used to evaluate the virulence of various strains, including those obtained from southern Brazil.

RPE transplants have been studied in some detail by the LI. One accomplishment is the establishment

of a reliable method for studying rejection phenomena of RPE cells. The group also has pursued its long-term interest in experimental uveitis models. The evaluation of various systems has resulted in further elucidation of the toleragenic state induced with the feeding of uveitogenic antigens. In parallel with these studies is an ongoing randomized masked study that will more fully evaluate the usefulness of S-antigen feeding in patients with intraocular inflammatory disease.

During the past year the group also has continued working on the development of a gene therapy approach for gyrate atrophy. The work to date has shown the feasibility of this approach; various studies have been designed to develop optimum ways by which this gene can be transfected into cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00065-16 OSD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Physiological Studies of the Primate Visual System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Francisco M. de Monasterio M.D., D.Sc. Medical Officer OSD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Office of the Scientific Director

SECTION

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project involves study of the physiological organization of neurons of the visual system of primates, with emphasis on the chromatic properties of color-opponent ganglion cells and of cells from the lateral geniculate nucleus and the primary visual cortex of macaques.

Project Description

Objectives

The purpose of this project is to study the neural organization underlying the processing of visual information at different levels of the primate visual system.

Methods

This research includes intracellular and extracellular recordings from single neurons, extracellular recordings of mass responses, computer video stimulation, and tangent screen chromatic and spatial stimulation.

Major Findings

The studies have been resumed only recently, after underground construction work ended both along the wall separating the laboratory rooms from the street and in a nearby building across the street. The construction work made microelectrode recordings from single neurons almost impossible due to the nearly continuous shaking of the ground, which was transmitted to the recording system despite attempts at vibration isolation. In addition, street digging by heavy machinery resulted in damage to the computer hard disks.

The studies involve an attempt of simultaneous recordings from both ganglion cell axons and geniculate cells receiving input from such axons to examine spectral property transformations between these two levels of the visual pathway.

Significance to Biomedical Research and the Program of the Institute

Numerous behavioral, psychophysical, and electrophysiological studies show that the visual performance and characteristics of macaques and humans are extremely similar to one another. An understanding of nonhuman primate physiology provides a useful animal model for human visual function.

Proposed Course

These studies will be continued.

NEI Research Program

Strabismus, Amblyopia, and Visual Processing—Visual Processing and Amblyopia (Structure and Function)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00122-13 OSD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Anatomical Studies of the Primate Visual System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Francisco M. de Monasterio M.D., D.Sc. Medical Officer OSD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Office of the Scientific Director

SECTION

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project involves study of the anatomical properties and organization of cells in the visual system of primates, with emphasis on the retina and the visual cortex. The studies include (1) the anatomical association of outer-retinal cells selectively stained with tissue-reactive dyes and (2) the pattern distribution of cones in the retinas of human donors.

Systematic study of the anatomical association at the light-microscope level of blue cones and horizontal and bipolar cells selectively stained by several tissue-reactive dyes in the macaque retina continues. The results have provided information on the probable retinal circuitry of the blue-sensitive cone pathway of primate retina.

Retinal cone studies of eyes from human donors also continue. Evidence of a cone population with a point pattern resembling that of blue-sensitive cones selectively stained by tissue-reactive dyes has been obtained in quickly fixed, well-preserved retinas of some donors with a clinical history of diabetes. Cone density studies are being conducted on the retinas of macaques and human donors of various ages to resolve issues on the degree of primate photoreceptor losses with aging.

Project Description

Objectives

This project was designed to study the anatomical properties and neural organization of the primate visual system.

Methods

This project involves retinal histological processing, intravitreal injection of dyes, computer modeling and spatial statistical analyses of point and area patterns, silver staining of cells and myelin, histological processing of the cerebral cortex, deoxyglucose labeling, autoradiography, and cytochrome oxidase labeling.

Major Findings

1. Human donor retinas fixed within 3 hours or less of time of death show a cone population with a point pattern distribution resembling that of the cones identified as blue-sensitive ones (i.e., absence in the central most region of the fovea, a peak density in the parafovea, and a regular though slightly disordered spacing in which stained cones are separated by two to three unstained cones) in donors with a reported longstanding clinical history of diabetes. This finding is consistent with clinical and psychophysical observations of a dysfunction of blue-sensitive cones in diabetic retinopathy. Fixation of the donor retinas in as short a time as possible after death continues to be a major obstacle in the obtaining of well-preserved material from a sizable number of cases.

2. Thin serial sections of macaque retinas, stained with a tissue-reactive dye that selectively stains blue-sensitive and some post receptoral cells, are being examined systematically by light microscopy to trace the anatomical relationship between selectively stained blue cones, H1 horizontal cells, and blue-cone bipolar cells of the more peripheral macaque retina. Data obtained so far provide evidence that two or three blue cones are commonly contacted by blue-cone bipolar cells in peripheral retina, whereas a single blue cone is typically contacted by a single blue-cone bipolar cell. These results indicate that the blue-sensitive cone system behaves in a manner similar to that of the so-called midget cell system, with increasing retinal eccentricity.

3. A modification of the tissue-reactive staining technique used to label blue-sensitive cones of primate retina has provided initial results of a sublabeling of the "unstained" cones into two apparently distinct populations. Because these populations may represent the green-sensitive and red-sensitive cones, several approaches are being tried to enhance such a labeling, which, if successful, would permit a direct observation of the point pattern of all three primate cone types.

Significance to Biomedical Research and the Program of the Institute

Information on the anatomical properties of blue-sensitive cones is important not only to the functional properties of these cones investigated in different basic disciplines but also to the clinical research and diagnosis of acquired retinal disease. The data obtained from the eyes of diabetic human donors are particularly promising in this respect.

Proposed Course

These studies will be continued.

NEI Research Program

Retinal and Choroidal Diseases—Fundamental Processes and Retinal Disorders (Retinal Organization, Neurotransmission, and Adaptation)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00135-21 OSD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemistry of Retina and Pigmented Epithelium in Health and Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Helen H. Hess M.D. Medical Officer (Research) OSD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Office of the Scientific Director

SECTION

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Effects of nutrition, oxidation, and other environmental factors (light intensity or darkness) on incidence and progress of posterior subcapsular opacities (PSO) associated with genetically influenced retinal degeneration are being studied in pink-eyed Royal College of Surgeons (RCS) rats, in which rod photoreceptor outer segment debris accumulates secondary to a phagocytic defect in retinal pigmented epithelium (RPE). Preoxidation in polyunsaturated fatty acids in debris led to water-soluble toxic aldehydes, detectable in the vitreous and toxic to lens cells and membranes. Dystrophic rats fed a natural ingredient diet (NIH-07) were highly sensitive to retina light damage, beginning at an intensity of 1-4 footcandles (FC), and 27% of the rats developed mature cataracts by 5-12 months. Rhodopsin bleaching is essential for retina light damage and PSO. *In vitro*, free retinaldehyde has been shown to be a photosensitizer to generate singlet oxygen, an extremely damaging oxidant for both lipids and proteins, and this also may occur *in vivo*.

A study of effects of environmental lighting on incidence of bilateral mature cataracts in pink-eyed RCS rats fed the natural ingredient diet (NIH-07) has been completed. Incidence of bilateral cataracts was 5% in rats reared in 1-4 FC of cyclic light but was 25% in rats reared in 10 FC constant light, 70% in 25 FC constant light, and 100% in 22- to 28-day-old rats given high-intensity light (700 FC) for 48 hours.

In RCS rats reared at 1-4 FC, a purified diet (AIN-76A) fortified with antioxidants (0.4% β-carotene + 0.01% BHT) prevented PSO and mature cataracts. Currently a diet containing additional antioxidants (1,000 mg/kg diet of vitamin C and 150 mg/kg vitamin E) retarded retinal degeneration during the time the cataracts would have had their onset (23-53 postnatal days) if NIH-07 had been fed. A study using higher concentrations of vitamin E has been completed, but the histopathological evidence in the retina has not been evaluated.

Project Description

Additional Personnel

J. Samuel Zigler, Jr.	Ph.D.	Chief, LMOD, NEI
Joseph J. Knapka	Ph.D.	Nutrition Consultant, Veterinary Research Program (VRP), National Center for Research Resources (NCRR)
Dennis Bernard	M.S.	Nutritionist, VRP, NCRR

Objectives

This project is designed to study the biochemical and bionutritional relationships between lens, retinal photoreceptors, retina, retinal pigment epithelium (RPE), and biological fluids in health and disease. It also involves exploring the possibilities for slowing the rate of retinal degeneration and preventing lens opacities and mature cataracts, which are often associated with retinal degeneration in rats and humans. Diseases in which the RPE may be involved are of particular interest.

Methods

The Royal College of Surgeons (RCS) rat is being studied as an animal model of hereditary retinal degeneration that results from a defect in the RPE as well as a type of cataract that is secondary to retinal degeneration. Bionutrition is being used as a tool to combat lipid peroxidation in the RCS rat retina and to prevent water-soluble toxic aldehyde byproducts from reaching and damaging the lens. The RCS cataract is not genetic, because the mutant gene is expressed not in the lens but in the RPE; it is instead an outcome of environmental risk factors of both internal and external origin. Thus, the RCS rat is a living laboratory, and the cataracts are susceptible to orchestration by varying risk factors and preventive measures.

Defined diets are prepared and fed to congenic affected and unaffected RCS rats in controlled experiments. The diets are fed to young breeding pairs prior to producing their first offspring and to their offspring after weaning, so that the experimental animals will have received their diets from conception to date of observation. Clinical findings are recorded after indirect ophthalmoscopic and biomicroscopic slit-lamp examination. Postmortem examinations of the eye include dissecting microscopy and light microscopy of stained specimens. At appropriate times, photography is used to record *in vitro* or *in vivo* data. Analytical methods include flameless atomic absorption, standard biochemical assays by spectrophotometry and fluorometry, and separation procedures. Special environmental lighting conditions are employed to determine their histopathological effects on the retina and the lens.

Major Findings

1. Previous work had yielded data on the incidence of bilaterality of mature cataracts in the pink-eyed, tan-hooded retinal dystrophic RCS rat under different intensities and duration of light, except for the standard conditions of cyclic light at 1-4 footcandles (FC) inside the cages. The omission occurred because the early data were obtained during a collaborative agreement in which any rat that developed a single cataract was sent for examination, and such rats could not be returned to observe bilateral occurrence. When our animal room was changed in location and in type of lighting (i.e., incandescent instead of fluorescent), it seemed wise to determine (1) whether incidence of cataract was the same and (2) the incidence of bilaterality. The diet in all these experiments on bilaterality was the standard natural ingredient diet (NIH-07). This diet contains all nutrients required by rats but permits the cataracts to occur.

The results showed the same incidence of cataract in incandescent as in fluorescent lighting (27%); 5% of the rats had bilateral cataracts by 1 year of age. In the previous studies, the incidence of bilaterality was greater in rats exposed to constant light: (a) 10 FC from 3 weeks to 1 year (25%), (b) 25 FC from 3 weeks to 1 year (70%), and 700 FC in 65-day-old rats in a short-term experiment of 2 days (100% of 15 rats). It seems possible that the 48-hour exposure at 700 FC could be reduced by using a program of short alternating exposures to light and darkness to produce the same percentage of cataracts and bilaterality, since this regimen has been shown to produce light damage in the retina. Thus, such experiments might be completed within the veterinary requirement of holding a rat for only 24 hours after removing it from the animal facility and also reduce the stress to the animal.

At 700 FC exposure for 48 hours, bilateral mature cataracts also were produced in congenic control RCS rats (15 of 15 rats), but the lag time before appearance of the cataracts was greater than in dystrophic rats (13-15 months, as compared to 9-12 months). Furthermore, cataracts only occurred if the light exposure was preceded by a long period of dark adaptation (18 days). If short exposures to light and darkness could be substituted in these experiments, this possible model of age-related cataract could be pursued further.

The requirement for long dark adaptation for production of mature cataracts in control RCS rats is consistent with our hypothesis that the cataracts are secondary to retinal light damage in both dystrophic and control rats. Long dark adaptation of normal retina increases the content of rhodopsin by 50%, to parallel the situation in dystrophic rats, in which the retinal content of rhodopsin is 70-100% greater than normal because of accumulation of rod outer segment debris from failure in phagocytic activity of the mutant RPE.

In vitro, retinaldehyde has been shown to act as a photosensitizer to generate singlet oxygen, a highly energetic oxidant for polyunsaturated lipids, as well as proteins. In retinas with a high rhodopsin content, the retinaldehyde released by bleaching may exist in the free state long enough to act as a sensitizer to generate singlet oxygen. In accord with the hypothesis, lens opacities would be initiated by water-soluble toxic lipid peroxidation products from degenerating retina, carried through the vitreous to attack membranes of lens cells and fibers. The prevention of cataracts by antioxidants would begin in the retina, with quenching of singlet oxygen by vitamin E and beta-carotene, and continue as these and other antioxidants combat secondary products of peroxidation.

2. Antioxidant diets that prevent cataracts in pink-eyed RCS dystrophic rats have the effect of retarding retinal degeneration. None of the diets we have tried stops the degeneration, but when a certain degree of retardation is achieved, the lens is protected. Last year we studied the AIN-76A purified diet, which contains twice the normal concentration of all the minerals in the AIN mineral mix plus 0.4% beta-carotene and 0.01% BHT, as well as 1,000 mg/kg vitamin C and 150 mg/kg vitamin E. After the rats consumed this diet, histopathological examination showed retarded retinal degeneration during the time the cataracts would have had their onset (23-53 postnatal days) if the NIH-07 diet had been fed. This year we fed that same diet containing increased concentrations of vitamin E to explore whether retinal degeneration can be delayed further. The eyes from rats of different ages have been fixed for histopathological study, but the results are not yet available. The dystrophic retina is extremely sensitive to light damage and to peroxidation of its lipids. This sensitivity, a fundamental part of the pathophysiology in the RCS rat, makes it more vulnerable to the defect of the RPE; furthermore, it may provide a clue to the underlying disease.

Significance to Biomedical Research and the Program of the Institute

The program goal of preventing posterior subcapsular cataracts (PSC) in RCS rats has been achieved, and the goal of slowing the rate of retinal degeneration has been advanced. When the retinal degeneration is slowed through 55 postnatal days, the cataracts are prevented. These results were obtained using bionutrition with a purified diet supplemented with antioxidants. PSC occurs in many varieties of human hereditary retinal degeneration known as retinitis pigmentosa (RP), as well as in so-called age-related cataracts and in some persons treated with steroids or exposed to short-wave radiation. None of the well-known types of human RP appears to show the problem of RPE phagocytosis found in the RCS rat; however, the number of types of RP appears to be very great, and many have not been explored for this phenomenon.

Cataract is a predominant cause of vision loss and blindness in the United States and in the world. Effective prevention in humans has not been developed; the only treatment is removal of the opaque lens. In the United States, the surgical treatment is safe, and intraocular lens replacement is highly successful. However, the annual cost of cataract surgery totals $2 billion and will continue to rise as the population ages. Cataract surgery, therefore, has been targeted for reduction as an important cost-saving plan, and prevention or slowing of cataract development has become imperative.

Among the known risk factors for cataractogenesis are ocular characteristics of the RPE and iris color, as well as exposure to sunlight and ultraviolet (UV) radiation. In humans, these and other risk factors are difficult to control and quantitate. In

RCS rats, however, the existence of both pink-eyed and black-eyed dystrophic and congenic control strains provides for control of ocular characteristics, and artificial illumination in the animal room provides the equivalent of sunlight and known UV radiation. In the RCS rat, the cataracts are secondary to the retinal degeneration, in which peroxidation of polyunsaturated fatty acids leads to water-soluble toxic aldehydes that are carried through the vitreous to the back of the lens, where they can damage the cell membranes of lens cells and fibers. Principles involved in this example of cataractogenesis may have relevance to some of the types of human cataract, including factors in slowing or preventing cataracts and retinal degeneration.

An initiative in the National Institutes of Health (NIH) Strategic Plan is the prevention or delay of cataract through nutrient-specific dietary intervention. Vitamins E and C and beta-carotene, as well as the antioxidant-associated trace minerals zinc and selenium, are to be tried in populations where diets are considered to be inadequate.

In an animal model, there is total control over the content and intake of nutrients, whereas this is difficult or impossible in a human setting. Our diets are fed to the parents prior to conception, to the female during pregnancy and in the lactation period, and to the experimental offspring during its entire life. In RCS rats, we employed a purified ingredient diet supplemented with twice the usual concentrations of normal minerals (including zinc and selenium), vitamins E and C, and beta-carotene. With this diet, the cataracts were totally prevented, and retinal degeneration was delayed through the time when the cataract would have had an onset if a standard natural ingredient rat diet had been fed. With the natural ingredient diet, cataract incidence was 27% in this rat strain under standard light conditions (cyclic light giving 1-4 FC intensity inside the cage). The percentage of cataracts seen with this diet increased with the light intensity, whereas rearing in darkness prevented cataract.

Proposed Course

Histopathological effects of feeding a high vitamin E antioxidant diet to the pink-eyed dystrophic rat will be evaluated to determine whether the retinal degeneration has been slowed further, beyond 55 postnatal days. Histopathological material from all previous rats with mature cataracts will be examined to compare the populations of lens epithelial cells. As pink-eyed, tan-hooded dystrophic breeders become available from the NIH foundation colony, lens epithelial cell whole mounts will be prepared to compare the numbers of cells in lenses with and without cataract.

Until now, studies of the mutant autosomal recessive rdy gene on Chromosome 3 of the RCS rat have not been feasible because the rat genome had not been well studied. However, knowledge of the rat genome has been proceeding apace, and a collaborative study to locate this gene, which appears to be involved in RPE phagocytosis, may soon be possible.

NEI Research Program

Cataract—Pathogenetic Mechanisms

Retina—Retinitis Pigmentosa and Other Inherited Disorders

Laboratory of Immunology

Report of the Chief, Laboratory of Immunology

Robert B. Nussenblatt, M.D.

The Laboratory of Immunology has finished its seventh year. Sections of the Laboratory include Immunology and Virology, headed by Dr. John J. Hooks; Experimental Immunology, headed by Dr. Igal Gery, who is also the Deputy Chief of the Laboratory; Immunoregulation, whose acting head is Dr. Rachel Caspi; Experimental Immunopathology, whose head is Dr. Chi-Chao Chan, and Clinical Immunology, whose acting head is Dr. Marc de Smet. For the recently formed Section on Molecular Biology, I serve as the acting head of an interdisciplinary group.

Section on Clinical Immunology

The Section on Clinical Immunology has continued to focus on major questions of clinical relevance. Studies centering on the evaluation of the use of the Molteno glaucoma implant and 5-fluorouracil combined with trabeculectomy continue. Final results still have not been obtained in this randomized long-term endeavor. On the basis of our observations, we are rather optimistic about the outcome for the long-term efficacy of the Molteno implant, although scientific evaluation of the results is still needed.

The Section continued its study of patients with AIDS (acquired immune deficiency syndrome), in collaboration with the National Institute of Allergy and Infectious Diseases. Evaluation of the safety of administering anticytomegalovirus (anti-CMV) hyperimmunoglobulin to patients at risk for CMV retinitis was concluded. A randomized study to evaluate a slow-release implant filled with gancyclovir is being actively tested in AIDS patients with CMV retinitis. These implants, placed directly into the eye through the porous plana, are calculated to release small but therapeutically effective amounts of gancyclovir over an 8-month period. This randomized study may yield information about an important alternative to systemic anti-CMV therapy for patients who cannot tolerate the intravenous infusions or who possibly do not have a specific indication for systemic therapy. Recruitment has been brisk, and we hope that results will be obtained within the next calendar year.

Also continuing are pediatric AIDS studies in which children are evaluated for the incidence of ocular infection. This study is done in conjunction with Dr. Philip Pizzo and his National Cancer Institute laboratory.

The Section also has been particularly interested in the development of immunosuppression. A randomized masked study to look at the effectiveness of oral tolerization has been in progress this past year. The research group is attempting to evaluate the usefulness of S-antigen (S-Ag) given per os in the induction of tolerance in uveitis patients. Initial observations in a pilot study have demonstrated a positive therapeutic response to feeding. The aim is to complete this randomized study within the next year and to have final results shortly thereafter.

Section on Molecular Biology

To acquire a better understanding of the approaches to gene therapy, this group has focused on regulation of the ornithine-δ-aminotransferase (OAT) gene *in vivo* as well as on genetic modification of somatic cell lines mediated by recombinant retroviruses. Dr. Moncef Jendoubi placed human OAT cDNA under the control of the enhancer-promoter regulatory elements derived from the Moloney murine leukemia virus long-terminal repeat, then transfected this construct into a safe packaging cell line (GP+E-86) to produce provirus particles. Supernatant from the ecotropic OAT producers of cell lines were used to transduce mouse embryonal fibroblasts as well as stem cells. The recombinant retrovirus transferred the OAT gene to the recipient cells, which produced an immunoreactive OAT. Northern blot analysis confirmed the presence of an OAT transcript in the transduced cell lines, even after a long time *in vitro*.

The Human Genome Group was established, and several individuals from various parts of the Laboratory have participated. The major goal is the development of a particular form of gene therapy in the treatment of gyrate atrophy. This gene therapy would entail use of the OAT gene. The work has shown that this gene can be transfected and has potential for use in such therapy. We hope that in the not too distant future this therapy will become a reality at the National Eye Institute.

Section on Immunoregulation

This Section has maintained interest in the development and study of animal models of experimental ocular autoimmune disease. The work has centered particularly on the characterization of the murine experimental autoimmune uveitis (EAU) model because the mouse offers some very important advantages over other rodent models of uveitis. In addition, the group has been actively involved in the establishment of antigen-specific T-cell lines and clones, which permit investigators to identify and characterize cells capable of inducing ocular immunomodulation.

This year the group has shown that the severity of inflammation and tissue damage in athymic rats is correlated with the proportion of lymphocytes in the intraocular infiltrate while the infiltrate in euthymic rats was predominantly lymphocytic with fewer monocytes and even fewer neutrophils. The sparse infiltrate in athymic rats was largely monocytic and had a relatively high proportion of neutrophils and eosinophils. It is interesting that reconstituted animals had an intermediate histological picture. These results indicate that recruited nonspecific T cells play a very major role in the pathogenesis of disease.

Collaboration with Dr. Charles Egwuagu continues in studies of the T-cell receptor (TCR) genes of cell lines and clones at the molecular level. Data collected to date indicate that TCR (variable region gene) usage in uveitis differs from that reported for other autoimmune diseases; it also may be more heterogeneous. It is interesting that recent experiments confirm our group's initial observations that TCR Vβ8.2 may be a pathogenic clonotype in S-Ag-induced uveitis, whereas TCR Vβ8.3 may be one of the pathogenic clonotypes in uveitis induced by interphotoreceptor retinoid-binding protein (IRBP). These findings are exceptionally important because of their impact on the development of therapeutic strategies that would specifically target pathogenic cells via the T-cell receptor.

Using a B10.A strain of mice, the group has developed a pathogenic T-cell line specific to whole IRBP. Such cell lines are interesting for multiple reasons, including the fact that the TCR profile of the line changes with time in culture. There appears to be progressive enrichment in the Vβ8.2 and Vβ8.6 TCR-expressing cells. In the mouse this would suggest that Vβ8.2 and Vβ8.6 represent pathogenic clonotypes in IRBP-induced uveitis.

Section on Experimental Immunology

Continuing its long-term investigation of the pathogenesis of inflammatory eye diseases, this Section has found that the induction of EAU by bovine IRBP in the Lewis rat involves a unique immunologic relationship. Lymphocytes sensitized against the immunodominant and uveitogenic determinant of IRBP do not recognize the rat homolog of this determinant; rather, they are stimulated by other peptides of the rat IRBP. In this description of the phenomenon, a surrogate peptide determinant of an organ-specific antigen is used to initiate an autoimmune pathogenic response.

In addition, the group has been actively involved in the S-Ag feeding oral tolerance studies. They have overcome the inaccessibility of large amounts of human S-Ag by using recombinant DNA technology to produce human S-Ag in *Escherichia coli* bacteria.

During the past year the group has evaluated the novel immunomodulator linomide, which was found effective in inhibiting EAU development in rats and mice actively immunized with S-Ag or IRBP. On the other hand, treatment with linomide had no effect on the development of EAU adoptively transferred by presensitized lymphocytes. These findings suggest that linomide would probably not be useful for the treatment of uveitis in humans.

Section on Experimental Immunopathology

This new Section had an extremely productive year. Immunohistochemistry and *in situ* hybridization have been used to identify and topographically localize immunocompetent cells and analyze the alteration of surface markers on ocular resident cells and their cytokines in experimental uveitis models as well as in ocular tissues. The T-cell dominance of the response remains one of the major factors that is seen again and again. Moreover, the migration of inflammatory cells from the vessels into the target appears to be directed by adhesion molecules that can be expressed on the vascular endothelium as well as other resident cells in the eye.

The group also has evaluated specimens from human ocular tissues with various diseases, including uveitis, retinal disease(s), conjunctival and corneal diseases, metabolic genetic diseases, and tumors. The group's demonstration of the presence of a major lens protein, αB-crystallin, in retinoblastoma suggests that αB-crystallin is involved in tumor growth and/or is a marker for general oncogenic stress in retinoblastoma.

Cell adhesion studies remain an area of great interest. The studies performed this year demonstrate that monoclonal antibodies against intercellular adhesion molecule 1 (ICAM-1) and its counterreceptor, lymphocyte function-associated antigen 1 (LFA-1), inhibit the development of EAU in mice. The group also has demonstrated that cell adhesion molecules are important for both antigen sensitization and inflammatory cell infiltration into the eye.

Section on Immunology and Virology

This Section has continued to emphasize its interest in the study of the retinal pigmented epithelial (RPE) cell. The use of monoclonal antibodies has helped in identifying a 67-kD protein that appears to be an RPE-specific epitope. Its sequence homology is similar to that of the intermediate filament of protein. The group also has demonstrated that inflammatory mediators such as lipopolysaccharide, tumor necrosis factor (TNF-α), and interleukin 1 (IL-1) induce interleukin 6 (IL-6) gene expression and secretion by human RPE cells. This effect also is noted to be synergistic with interferon gamma (IFN-γ). RPE cell transplantation continues to be a major area of interest for this group as well.

During the past year the group developed a reproducible model for ocular toxoplasmosis. The induction of this model has provided the Laboratory with an opportunity to evaluate strain virulence. A strain of toxoplasmosis virulent in humans and isolated from Brazil was shown to be particularly virulent in a mouse model. Studies have begun to evaluate the mechanisms involved in toxoplasmosis cyst formation as well as attempts to counteract the infection.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00279-02 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of Immunosuppressants for the Treatment of Uveitis in Animal Models

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	François G. Roberge	M.D.	Visiting Scientist	LI, NEI
Others:	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI
	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Dan Martin	M.D.	Senior Staff Fellow	LI, NEI
	Margaret Cheung	M.D., Ph.D.	Senior Staff Fellow	LI, NEI
	David Parks	M.D.	Senior Staff Fellow	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Clinical Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goals of the project are to acquire a better understanding of the immunopathogenic mechanism of noninfectious intraocular inflammatory diseases (uveitis) and to develop treatment and prevent the complications associated with these diseases. This past year we studied two specific aspects of the application of the new noncytotoxic immunosuppressant rapamycin (RAPA) in the treatment of uveoretinitis: (1) evaluation of the synergistic effect of RAPA with cyclosporine A (CsA) or dexamethasone (Dex) and (2) evaluation of the effect of RAPA on complications of uveitis, fibrosis, and intraocular membrane formation. We also evaluated the role played by nitric oxide (NO) in anterior uveitis.

We demonstrated that combining RAPA with CsA or Dex can inhibit experimental uveitis *in vivo* at greatly reduced doses of each drug, compared with the doses necessary when these three drugs are used singly. We showed that RAPA also inhibits proliferation of human fibroblast and retinal pigment epithelial (RPE) cells, indicating the advantage of using RAPA in the treatment of uveitis, in which fibrosis and membrane formation are common complications. We also demonstrated that NO is an important mediator of endotoxin-induced anterior uveitis.

Project Description

Additional Personnel

Bruce Pfeffer	Ph.D.	Senior Staff Fellow, LRCMB, NEI

Clinical Protocol Numbers

91-191
91-187

Objectives

The immunosuppressive treatment of autoimmune disease such as uveoretinitis usually has to be sustained for a long period of time. To avoid treatment complications as much as possible, we try to develop treatment using noncytotoxic drugs. In particular, we seek to develop combination therapy that minimizes the toxicity of each drug while maximizing the beneficial effects. Previously we had demonstrated by *in vitro* studies that the combinations of rapamycin (RAPA) with cyclosporine A (CsA) and RAPA with dexamethasone (Dex) had a synergistic effect on the inhibition of the proliferation of retinal antigen-primed lymphocytes. This year we tested these results in the *in vivo* rat model of experimental autoimmune uveoretinitis (EAU). We evaluated the effect that RAPA could have on the fibrosis and membrane formation in the eye following inflammation.

We also have studied the role of nitric oxide (NO) in a model of anterior uveitis induced with endotoxin (lipopolysaccharide). NO is the oxidation product of one of the guanidino nitrogens of L-arginine. The reaction is catalyzed by two different forms of the enzyme nitric oxide synthase, which have specific tissue distributions. NO is produced after stimulation with endotoxin. The synthesis of NO can be competitively blocked with L-arginine analogues such as N^G-nitro-L-arginine methyl ester (L-NAME). The inhibition is enantiomer restricted, the D form of analogues being inactive; it is also fully reversible with an increased concentration of L-arginine. We used the specificity of L-NAME inhibitory action to evaluate indirectly the role of NO in endotoxin-induced uveitis (EIU).

Methods

EAU was induced by immunization with S-antigen in Hunter's adjuvant. Treatment was given for 14 days, starting on Day 7, and the experiment terminated on Day 28 after immunization. RAPA was delivered by continuous intravenous (i.v.) infusion by means of a miniosmotic pump implanted in the abdominal cavity. CsA or Dex was given by intramuscular (im) injection once a day. The disease was evaluated by histopathology.

Two human RPE lines, between passages 4 and 8, and human skin fibroblasts were used. Proliferation assays were done in quadruplicate in Dulbecco's modified Eagle's medium/F12 medium, in microtiter plates at 6×10^3 cells per well. Proliferation was measured by the incorporation of ^{3}H-thymidine, added after 24 hours for 18 hours of incubation. Stimulants were 5% fetal bovine serum; IGF-1 (50 ng/ml); and aFGF, bFGF, EGF, and PDGF (10 ng/ml each). RAPA, CsA, FK 506, or solvent alone was added at the initiation of culture in concentrations ranging from 10^{-6} to 10^{-11} M. Toxicity of the drugs was assessed by a quantitative tetrazolium-based colorimetric assay.

Uveitis was induced in rats with subcutaneous LPS. The animals were treated with L-NAME, an L-arginine analog acting as a specific inhibitor of NO synthesis. Ocular inflammation was evaluated by measuring the protein concentration and leukocyte number in the aqueous humor of one eye and by histopathology of the contralateral eye.

Major Findings

We found that we could effectively inhibit EAU by using a combination of RAPA with CsA in which the doses were reduced by factors of 10 and 5, respectively, compared with the doses necessary when the drugs were used alone. Similarly, in the RAPA with Dex combination, the doses were reduced by fourfold and fivefold, respectively.

We found that RAPA could significantly inhibit the proliferation of human retinal pigment epithelial cells as well as human fibroblasts, thus RAPA could be indicated for patients in whom uveitis is complicated by fibrosis and retinal membrane formation.

We demonstrated for the first time that NO is a crucial mediator of EIU and that its inhibition may, in the future, become an avenue of therapy for anterior uveitis.

Significance to Biomedical Research and the Program of the Institute

Our study on combination therapy for EAU has shown that the observed *in vitro* synergy between

RAPA and CsA and between RAPA and Dex was effective *in vivo*. Such combination therapy, if applied to humans, could significantly reduce the toxic side effect of the treatment while allowing good control of the disease. In addition, RAPA could benefit patients for whom uveitis is complicated by intraocular fibrosis and membrane formation, two complications that are often responsible for a large part of vision loss.

The newly discovered role for NO in anterior uveitis could lead to improved therapy by finding inhibitors of the production of NO in the eye.

Proposed Course

In the future, we intend to develop a clinical trial for the treatment of uveitis with RAPA in humans. A protocol has been developed and will be submitted to the producer of the drug.

The study concerning NO and uveitis will be developed in the direction of identifying the cells responsible for the effect found in the eye. In addition, different types of NO inhibitors will be tested to find the one(s) most appropriate for uveitis therapy.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

Li Q, Lopez JS, Caspi RR, Roberge FG, Nussenblatt RB, Kador PF, Chan C-C: Suppression of S-antigen induced experimental autoimmune uveoretinitis in Lewis rats by oral administration with CGS-13080, a thromboxane synthetase inhibitor. *Exp Eye Res* 57:601-608, 1993.

Roberge FG, Kozhich A, Chan C-C, Martin DF, Nussenblatt RB, de Smet, MD: Inhibition of cellular transfer of experimental autoimmune uveoretinitis by rapamycin. *Ocular Immunol Inflam* 1:269-73, 1993.

Roberge FG, Xu D, Chan C-C, de Smet MD, Nussenblatt RB, Chen H: Treatment of autoimmune uveoretinitis in the rat with rapamycin, an inhibitor of lymphocyte growth factor signal transduction. *Curr Eye Res* 12:197-203, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00262-04 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of T Lymphocytes and Cytokines Involved in Experimental Autoimmune Uveoretinitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Charles E. Egwuagu	Ph.D., M.P.H.	Senior Research Scientist	LI, NEI
Others:	Igal Gery	Ph.D.	Head, Section on Experimental Immunology	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Rachel Caspi	Ph.D.	Visiting Associate	LI, NEI
	Rashid Mahdi		Biologist	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Experimental Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Experimental autoimmune uveoretinitis (EAU) is a T-cell-mediated autoimmune disease that serves as a model of human intraocular inflammatory diseases (uveitis). It is initiated in susceptible animals by immunization with retinal antigens, such as interphotoreceptor retinoid-binding protein (IRBP) or S-antigen (S-Ag). We had shown previously that Vβ8-expressing T cells accumulate in the retina during EAU. Because the Vβ8-expressing T cells comprise cells with distinct functional activities, we sought to identify the uveitogenic subpopulation by analyzing the T-cell antigen receptor (TCR) genes and the cytokines they secrete in the retina during EAU. Our results indicate that the Vβ8 response is highly dependent on the antigen used for disease induction: Whereas only Vβ8.2$^+$ T cells were found in the retinas of animals with S-Ag EAU, both Vβ8.2$^+$ and Vβ8.3$^+$ T cells were present in the retinas of rats with IRBP EAU. In terms of the pattern of cytokine production, both Th1- and Th2-like lymphokine profiles were observed, and the cytokine detected in the retina was found to be dependent on the antigen used for disease induction. Our current data suggest that the T-cell Vβ8 response in the retina during EAU is heterogeneous. Thus, despite the fact that there is a bias toward use of Vβ8$^+$ cells in the retina during EAU, a single anti-Vβ8 antibody cocktail may not confer full protection from uveitis because several autoantigens and an unknown number of immunopathogenic T-cell clonotypes may be involved in the induction of EAU.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

This project is aimed at determining the clonality of the T lymphocytes that mediate ocular autoimmune diseases. Identification of the pathogenic T-cell subset in experimental autoimmune uveoretinitis (EAU) is relevant to our goal of developing anti-T-cell receptor (TCR) therapies for the treatment of uveitis. Our effort during Fiscal Year 1993 focused on analyses of T cells present at the autoimmune site and the lymphokines they produce.

Methods

In situ, reverse-transcribed polymerase chain reaction (RT/PCR) was used to examine T-cell populations in the retinas of athymic and euthymic Lewis rats at different stages of EAU. We used the cDNAs generated to amplify transcribed genes that code for rat Vβ TCR chains, T-cell subset markers (CD4 and CD8), and cytokines associated with autoimmune pathology—interferon-gamma (IFN-γ), tumor necrosis factor (TNF-α), transforming growth factor (TGF-β), interleukin 2 (IL-2), and interleukin 6 (IL-6). Conventional recombinant DNA techniques such as Southern blot hybridization, PCR, cDNA construction, densitometry, and DNA sequencing were used to analyze the resultant PCR products.

Major Findings

Analysis of TCR Vβ8 repertoire induced by S-antigen (S-Ag) and interphotoreceptor retinoid-binding protein (IRBP).—We analyzed TCR genes expressed in response to immunopathogenic epitopes of human S-Ag or bovine IRBP by the PCR assay. We found that expression of Vβ8 genes in the retinas of S-Ag-immunized rats was restricted to Vβ8.2 TCR, while both Vβ8.2 and Vβ8.3 TCR isoforms were detected in IRBP-immunized rats. Vβ8.1$^+$ T cells were not detectable in the retinas of rats with either IRBP- or S-Ag-induced EAU.

Analysis of Vβ8 repertoire in adoptively transferred EAU.—In contrast to EAU induced by active immunization, all three Vβ8 subfamily members were detected in the retina in EAU induced by adoptive transfer of IRBP- or S-Ag-specific uveitogenic T cells. Skewing of the Vβ8 repertoire during EAU induced by active immunization points out a potential confounding factor in vaccination studies in which complete Freund's adjuvant is a component of the vaccine preparations. The earliest Vβ8$^+$ T cells detected in the retina were of the Vβ8.2 clonotype. This subpopulation was followed by Vβ8.3- and Vβ8.1-expressing clonotypes. Similar to the response to active immunization, the initial appearance of Vβ8.3$^+$ cells was subsequently followed by a rapid decline in the numbers of Vβ8.3$^+$ lymphocytes.

Analysis of cytokine production in the retina during EAU.—In adoptively transferred EAU, the cytokines in which production in the retina could be correlated with EAU pathogenesis are TNF-α, IFN-γ, and IL-6. However, the temporal appearance of these cytokines in the retina was dependent on the antigen used for eliciting the uveitogenic T cells.

Significance to Biomedical Research and the Program of the Institute

Our current data show selective accumulation of Vβ8$^+$ cells in the retina during EAU. However, the T-cell response is not oligoclonal, and distinct Vβ8 subfamilies were differentially activated by the autoantigens S-Ag and IRBP. Furthermore, the patterns of lymphokine production indicate that distinct T-cell subsets may initiate IRBP- and S-Ag-induced EAU. Taken together, these findings suggest that a single anti-Vβ antibody cocktail may not confer full protection from uveitis because several autoantigens and an unknown number of immunopathogenic T-cell clonotypes may be involved in the pathogenesis of EAU.

Proposed Course

Future analyses of uveitogenic T-cell clonotypes and the lymphokines they produce during EAU will be performed in nude rats. Because these rats do not normally produce T cells, we expect the intraretinal T-cell repertoire in these rats to be limited. This would allow for a more concise analysis of the identity of the relevant autoaggressive T cells involved in uveitis.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

Egwuagu CE, Bahmanyar S, Mahdi R, Nussenblatt RB, Gery I, Caspi R: Predominant usage of

Vβ8.3 T cell receptor in a T cell line that induces experimental autoimmune uveoretinitis. *J Clin Immunol Immunopathol* 65:152-160, 1992.

Egwuagu CE, Caspi R, Mahdi R, Gery I, Nussenblatt RB: Evidence for selective accumulation of Vβ8+ T lymphocytes in experimental autoimmune uveoretinitis induced by two different retinal antigens. *J Immunol* 151:1627-1636, 1993.

Mahdi RM, Caspi RR, Nussenblatt RB, Gery I, Egwuagu CE: Selective accumulation of $V\beta8^+$ T lymphocytes in EAU. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1144, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00280-02 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ectopic Expression of Interferon-Gamma in the Eyes of Transgenic Mice and Rats Induces Ocular Pathology and MHC Class II Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Charles E. Egwuagu	Ph.D., M.P.H.	Senior Scientist	LI, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI
	Ana B. Chepelinsky	Ph.D.	Head, Section on Regulation of Gene Expression	LMDB, NEI
	Jorge Sztein	D.V.M., Ph.D.	Visiting Associate	LI, NEI
	Rashid Mahdi		Biologist	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Experimental Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To investigate the consequences of ectopic expression of interferon gamma (IFN-γ) in the lens and how this lymphokine growth factor may influence the fate of cells committed to a particular differentiation state, we used the murine αA-crystallin promoter (αACry) to direct the expression of IFN-γ to the lens of transgenic mice. Two transgenic mouse lines were established using two distinct strains, Balb/c and FVB/N. In both the αACry-IFN-γ/Balb/c and αACry-IFN-γ/FVB/N transgenic mice, the essential histopathological features observed were very similar at all developmental stages studied (i.e., 12- to 18-day embryos, newborns, adults), suggesting that maldevelopment of ocular tissues of these mice resulted from αACry-IFN-γ expression. Recently we have generated transgenic rats using the αACry-IFN-γ cDNA construct, making our transgenic rats the first transgenic rats to be generated at the National Institutes of Health.

Constitutive expression of IFN-γ in the lens induced ocular pathology and aberrant major histocompatibility complex (MHC) class-II protein synthesis in several ocular tissues. These transgenic mice and rats provide powerful models for understanding the molecular pathways governing synchronized programmed differentiation of ocular tissues and for studying the linkage between aberrant MHC expression and predisposition to autoimmune diseases.

Project Description

Objectives

This project is designed to generate transgenic animals (rats and mice) that selectively secrete interferon-gamma (IFN-γ) in their eye tissues for use in studying the bioregulatory actions of IFN-γ in the eye. Because aberrant expression of major histocompatibility complex (MHC) molecules is an early event in a number of human autoimmune diseases and IFN-γ induces high levels of MHC class II protein biosynthesis, these transgenic mice and rats are ideally suited for studying (1) the effects of IFN-γ on the physiology of the eye and (2) the role of elevated MHC class II in predisposition to autoimmune diseases such as diabetes and uveitis.

Methods

Transgenic animals were generated according to standard transgenic mouse techniques; however, for the transgenic rats, adjustments in the hormone treatment regimen were necessary to obtain embryos used for microinjection of the construct. Transgenic and wild-type (WT) phenotypes were characterized by histologic examination of representative tissue sections. We used conventional recombinant DNA techniques—such as Southern blot hybridization, polymerase chain reaction, cDNA construction, densitometry, and DNA sequencing—to characterize DNAs and RNAs derived from WT and transgenic mice.

Major Findings

The most notable effects of IFN-γ in the transgenic mice include cataract, microphthalmia, blepharophimosis, microphakia, impairment of lens fiber formation, arrest of retinal differentiation, serous retinal detachment with the presence of macrophages in the subretinal space, persistent hyperplastic primary vitreous, and corneal vascularization. MHC class II mRNA levels were significantly increased in the transgenic eyes, and MHC class II proteins were expressed in the cornea, iris, ciliary body, choroid, lens, and retinal pigment epithelium.

Significance to Biomedical Research and the Program of the Institute

The generation of transgenic rats is a major technical accomplishment that now allows us to generate rats containing other DNA constructs. This is particularly important considering that several ocular diseases, such as uveitis and cataract, are better suited for study in the rat than in the mouse. Our data suggest that αACry-IFN-γ transgenic mouse ocular cells express functional IFN-γ receptors and that interaction of IFN-γ with its receptor induced biochemical and morphological changes in the transgenic eyes. These transgenic mice and rats provide a powerful model for understanding the molecular pathways that govern synchronized, programmed differentiation of ocular tissues and for studying the linkage between aberrant MHC expression and predisposition to autoimmune diseases.

Proposed Course

We intend to further dissect the molecular basis of IFN-γ actions in the eye, placing particular emphasis on the rat model. A major focus will be the study of transcriptional factors that mediate αACry-IFN-γ effects.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

Egwuagu CE, Sztein J, Reid W, Chan C-C, Mahdi R, Nussenblatt RB, Chepelinsky AB: Ectopic expression of gamma interferon in the eyes of transgenic mice induces ocular pathology and MHC class II gene expression. *Invest Ophthalmol Vis Sci*, in press.

Egwuagu CE, Sztein J, Reid W, Chan C-C, Mahdi R, Nussenblatt RB, Chepelinsky AB: Gamma interferon gene expression in the lens of transgenic mice directed by the αA-crystallin gene promoter. *Invest Ophthalmol Vis Sci* 34(4) (suppl):846, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00069-16 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immune Responses to Ocular Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Igal Gery	Ph.D.	Head, Section on Experimental Immunology	LI, NEI
Others:	Alexander Kozhich	Ph.D.	Visiting Fellow	LI, NEI
	Xiaowen Wu	M.D.	Visiting Fellow	LI, NEI
	Nancy Miller-Rivero	M.D.	IRTA Fellow	LI, NEI
	Barbara Vistica	B.A.	Microbiologist	LI, NEI
	Norman Chanaud III	B.A.	Special Volunteer	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI

COOPERATING UNITS *(if any)*

Biotechnology Unit, Laboratory of Cellular and Developmental Biology, National Institute of Diabetes and Digestive and Kidney Diseases (Joseph Shiloach, Ph.D.); Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA (David Hafler, M.D.); Department of Medicine, Hadassah Hospital, Jerusalem, Israel (Shimon Slavin. M.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Experimental Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.2	3.8	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Targeted at learning about the pathogenesis of inflammatory eye diseases grouped under the term "uveitis," this project continued to focus mainly on learning about ocular antigens that induce experimental autoimmune uveoretinitis (EAU), an animal model for uveitis in man, and on procedures that modulate this disease. Three major achievements of this study are as follows: (1) We found that the induction of EAU by bovine interphotoreceptor retinoid-binding protein (IRBP) in the Lewis rat involves a unique immunologic reaction in which lymphocytes sensitized against the immunodominant and uveitogenic determinant of this protein do not recognize the rat homolog of this determinant, but, rather, are stimulated by another peptide of the rat IRBP. This is the first description of a phenomenon in which a "surrogate" peptide determinant of an organ-specific antigen is used to initiate an autoimmune pathogenic process. (2) To overcome the inaccessibility of large amounts of human S-antigen (S-Ag), we have used recombinant DNA technologies to produce this human antigen in *Escherichia coli* bacteria. The recombinant human S-Ag was found to cross-react with native bovine S-Ag and to be similarly capable of inducing EAU in Lewis rats. In addition, the recombinant human S-Ag was used to identify the immunodominant peptide determinants of this antigen, i.e., the peptides recognized by lymphocytes sensitized against whole human S-Ag. Three regions of the molecule were found to harbor immunodominant determinants, with different peptides being selected as dominant in rats of different inbred strains. (3) A novel immunomodulator, linomide, was found to be effective in inhibiting EAU developing in rats and mice actively immunized with S-Ag or IRBP, respectively. This drug also inhibited the humoral and cellular immune responses in these animals. On the other hand, treatment with linomide had no effect on the development of EAU adoptively transferred by presensitized lymphocytes. The latter finding suggests that linomide cannot be useful for the treatment of uveitis in humans, in whom immunopathogenic processes are preactivated.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

Studies conducted during Fiscal Year (FY) 1993 were aimed at the following: (1) to further analyze the immune responses to the immunodominant and highly uveitogenic peptide 1181-1191 of bovine interphotoreceptor retinoid-binding protein (IRBP); (2) to prepare recombinant human S-antigen (rHumS-Ag) and test its immunological properties—(a) its immunogenicity and uveitogenicity in rats, (b) its cross-reactivity with bovine S-antigen (S-Ag), and (c) identification of the peptide determinants in its sequence that are immunodominant in various rat strains; and (3) to investigate the effect of linomide, a novel immunomodulator, on the development of experimental autoimmune uveoretinitis (EAU) in rats and mice.

Methods

Peptides were synthesized on an Applied Biosystems synthesizer 430A. Bovine S-Ag was extracted from frozen retinas by Dr. Joseph Shiloach (National Institute of Diabetes and Digestive and Kidney Diseases). rHumS-Ag was expressed in *Escherichia coli* according to the method described by H. F. Oettinger et al. (*J Neuroimmunol* 44:157-162, 1993) using a cDNA coding for human S-Ag. A large batch of the recombinant antigen was prepared by Dr. Shiloach and was extracted by 8 M urea. Conventional procedures were used for immunization of animals and for testing their immune responses and development of EAU. The affinity of peptides toward major histocompatibility complex (MHC) molecules on antigen-presenting cells (APC) was assessed by the capacity of the tested peptide to inhibit the binding of biotinylated bovine IRBP peptide 1181-1191 to adherent cells from syngeneic spleens. Linomide was provided by Dr. S. Slavin (Hadassah Hospital, Jerusalem). The drug was administered to experimental animals via the drinking water at 1 mg/ml.

Major Findings

Studies concerning IRBP peptide 1181-1191.—As indicated in our report for FY 1992, the rat homolog of bovine IRBP peptide 1181-1191 is immunologically inactive in the Lewis rat and is not recognized by lymphocytes sensitized to the bovine sequence. Since the bovine peptide is immunodominant and highly uveitogenic in Lewis rats (Sanui et al., *J Exp Med* 169:1947, 1989), lymphocytes sensitized against it must recognize a determinant of the rat IRBP molecule to initiate the pathogenic process of EAU. Our studies now show that this target determinant is likely to be the rat IRBP peptide 273-283: (1) sequence 273-283 of IRBP, which is extremely conserved, is identical in bovine and rat; (2) rat peptide 273-283 is recognized by lymphocytes sensitized against bovine peptide 1181-1191 and stimulates them to proliferate; and (3) moreover, rat 273-283 is superior to bovine 1181-1191 in its capacity to stimulate uveitogenicity in lymphocytes sensitized against the bovine peptide.

The lack of immunogenicity of the rat IRBP peptide 1181-1191 in Lewis rats was found to be related to the poor affinity of this peptide toward the MHC molecules on the APC of Lewis rats. (T lymphocytes recognize antigenic determinant only in the context of the MHC molecule.) The poor affinity of the rat peptide 1181-1191 was shown by its inability to competitively inhibit the binding of the bovine 1181-1191 to Lewis rat spleen cells. In line with the assumption that the lack of immunogenicity of rat peptide 1181-1191 in Lewis rats is due to its poor affinity to the Lewis MHC, we found that this rat peptide is immunogenic and uveitogenic in rats of the Buffalo inbred strain; the Buffalo MHC class II is $RT1^b$, while the Lewis MHC molecule is $RT1^l$. Moreover, bovine 1181-1191 was found to be nonuveitogenic and nonimmunogenic in the Buffalo rat.

Studies with rHumS-Ag.—Partially purified rHumS-Ag, expressed in *E. coli*, was examined for its immunological activities in rats. Our findings were as follows: (1) rHumS-Ag resembled bovine S-Ag (extracted from retinas) in its capacity to induce EAU in Lewis rats; the minimal dose of both preparations was 2.5 μg per rat (when injected in complete Freund's adjuvant, along with *Bordetella pertussis*). (2) There were moderate levels of cross-reactivity between the two preparations when tested by either antibody or cellular immune responses. (3) Lymphocytes from rats immunized with rHumS-Ag were used to identify the immunodominant peptide determinants of human S-Ag, namely the determinants against which the immune response is targeted in animals immunized with the whole antigen. When tested against 40 overlapping syn-

thetic peptides that extend the whole antigen sequence, sensitized lymphocytes from Lewis rats immunized with rHumS-Ag responded against peptides derived from three sequence regions: 61-80, 171-190, and 281-310. The highest lymphocyte response was observed against peptide 281-300, which is also uveitogenic in Lewis rats. On the other hand, we observed low proliferative responses with peptides 341-360 and 351-370, which are highly uveitogenic in the Lewis rat.

Testing the responses of rHumS-Ag-sensitized lymphocytes from rats of inbred strains other than Lewis revealed that the same three regions of human S-Ag provide the immunodominant peptides for the response of all tested strains, but we saw remarkable differences among the strains with regard to their response to individual peptides. Thus, the highest response of Buffalo rats was aimed at peptides 61-80 and 71-90; ACI rats responded mainly to peptides 71-90 and 181-200, while BN rats responded at the highest levels against peptides 71-90 and 291-310.

The effects of linomide in the EAU system.— Linomide (quinoline-3-carboxamide) is a unique immunomodulator that both enhances natural killer cell activity and suppresses autoimmune processes. Treatment with linomide inhibited the development of actively induced EAU: In mice, 11 of 15 controls had EAU versus none of the 15 in the treated group; in rats, 11 of 12 controls developed EAU versus 5 of 12 in the treated group. The disease developed later and was less severe in treated rats than in control rats. Linomide treatment also significantly suppressed humoral and cellular immune responses in both mice and rats. In contrast, however, treatment with linomide had no effect on the development of adoptively transferred EAU or on the immune responses in the recipient animals.

Significance to Biomedical Research and the Program of the Institute

1. Our findings with IRBP peptide 1181-1191 reveal for the first time a unique immunological system in which lymphocytes sensitized against an immunodominant peptide of a xenogeneic organ-specific protein do not recognize the autologous homolog but, instead, initiate the pathogenic autoimmune process by recognizing a "surrogate" peptide epitope of the autologous molecule. This unique phenomenon is made possible by the unusual four-fold structure of the IRBP molecule, allowing cross-reactivity between "repeats" on different regions of the molecule. In addition, our observations with this immunological system provide new evidence of the pivotal role of the affinity of a peptide toward the MHC in determining the immunogenicity of the peptide in animals using that MHC molecule.

2. The limited supply of human retinas has restricted in the past usage of the human S-Ag in the various immunological studies concerning the involvement of this retinal antigen in human diseases. Consequently the large majority of studies have been carried out with the bovine protein. The successful expression of immunologically active rHumS-Ag thus provides us with an essentially limitless supply of this antigen. It is expected that rHumS-Ag will become a useful tool for analyzing autoimmunity in uveitic patients. Moreover, rHumS-Ag may be the antigen of choice in clinical studies in which oral tolerance with retinal antigens will be used as a modality to modulate the pathogenic process of autoimmune-mediated uveitis.

3. Our study with linomide provides the first data concerning the effect of this immunomodulator on an autoimmune ocular disease. Moreover, our data indicate that, unlike its effectiveness in inhibiting immune responses of the afferent type, linomide has no effect on the efferent limb of the immune response. This new observation underscores the uniqueness of the mode of action of this compound, but it casts doubt on the future usefulness of linomide in clinical conditions.

Proposed Course

Our future efforts will focus on the following issues: (1) the relationship between the affinity of IRBP peptides to MHC molecules and the immunogenicity and uveitogenicity of these peptides in rats of various inbred strains and (2) analysis of the system in which feeding with uveitogenic antigens protects animals against the development of EAU and immune response toward these antigens. In particular, we will examine the capacity of rHumS-Ag to induce oral tolerance. We will probe the possibility of using this antigen in the treatment of patients with uveitis.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

Casper-Velu LE, Verougstraete C, Gery I, Nussenblatt RB: Ultrastructural changes of retinal vascular endothelial cells at the onset of experimental autoimmune uveitis, in Dernouchamps JP, Verougstraete C, Caspers-Velu L, Tassignon MJ (eds): *Recent Advances in Uveitis.* Amsterdam, Kugler Publications, 1993, pp 103-110.

Egwuagu CE, Bahmanyar S, Mahdi RM, Nussenblatt RB, Gery I, Caspi RR: Predominant usage of Vβ8.3 T cell receptor in a T cell line that induces experimental autoimmune uveoretinitis (EAU). *Clin Immunol Immunopathol* 65:152-160, 1992.

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Fujino Y, Li Q, Chung H, Hikita N, Nussenblatt RB, Gery I, Chan C-C: Immunopathology of experimental autoimmune uveoretinitis in primates. *Autoimmunity* 13:303-309, 1992.

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Kasner L, Chan C-C, Whitcup SM, Gery I: The paradoxical effect of tumor necrosis factor alpha (TNF-α) in endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci* 34:2911-2917, 1993.

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Suh EDW, Vistica BP, Chan CC, Raber JM, Gery I, Nussenblatt RB: Splenectomy abrogates the induction of oral tolerance in experimental autoimmune uveoretinitis. *Curr Eye Res* 12:833-839, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00288-01 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gene Therapy for Ocular Genetic Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Moncef Jendoubi	Ph.D.	Visiting Scientist	LI, NEI
Others:	Noriko Esumi	M.D., Ph.D.	Visiting Associate	LI, NEI
	Daniel H. Lacorazza	Ph.D.	Visiting Fellow	LI, NEI
	Luis J. Rivero	Ph.D.	Visiting Fellow	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Genetics and Molecular Immunology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.9	3.9	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goals of our main project are to get a better understanding of the physiological regulation of the ornithine δ-aminotransferase (OAT) and its regulation *in vivo*. In humans, the mutation of OAT leads to the degeneration of the choroid and retina, causing a gyrate atrophy disease. There is no treatment for this human genetic disease; the only feasible approach would be to submit the patient to gene therapy via modified somatic cell lines.

To accomplish this task, we are focusing on (1) the regulation of OAT gene *in vivo* and (2) the genetic modification of somatic cell lines mediated by recombinant retroviruses. With the idea of applying gene therapy, Moloney murine leukemia virus-based recombinant retrovirus vectors have been constructed. The human OAT cDNA was placed under the control of the enhancer-promoter regulatory elements derived from the Moloney murine leukemia virus long-terminal repeat. The construct was transfected into a safe packaging cell line, GP+E-86, to produce provirus particles. Supernatant from these ecotropic OAT producer cell lines was used to transduce mouse C57B1/6 embryonal fibroblasts and embryonic stem cells. We showed that the recombinant retrovirus transfers the OAT gene to the recipient cells, which produce an immunoreactive OAT. Northern blot analysis confirmed the presence of an OAT transcript in the transduced cell lines, even after a long period of time *in vitro*.

Project Description

Objectives

Ornithine δ-aminotransferase (OAT, L-ornithine:2-oxoacid aminotransferase, EC 2.6.1.13) is a nuclear-encoded mitochondrial matrix housekeeping gene that catalyzes the reversible transamination of ornithine to glutamate semialdehyde. Biochemical analyses have shown that OAT is synthesized as 49-kD precursor monomer cleaved to a 45-kD protein on entry into the mitochondria, where assembly into the active homohexameric form of the enzyme occurs. This enzyme is expressed constitutively at low levels in the liver and kidney and at much higher levels in the retina, where OAT function seems to be critical for vision.

Methods

In humans, a genetic deficiency of OAT results in gyrate atrophy (GA) of the choroid and retina—a rare, blinding chorioretinal degeneration with associated cataract, inherited as an autosomal recessive trait. Such OAT deficiency disrupts ornithine and arginine catabolism and results in a 10- to 15-fold accumulation of ornithine in all body fluids. In addition to their visual problems, some GA patients exhibit muscular dystrophy. Using a variety of techniques, including RNase A protection, single-strand conformational polymorphism analysis, and polymerase chain reaction, recent studies have shown that the unique OAT gene in GA patients contains frameshift, nonsense, and missense mutations that lead to the inactivation of the gene.

A diet low in arginine has been shown to possibly delay the onset of this disease, but this approach is difficult to follow and may not be applicable to all patients. Therefore, since no therapy has been shown to be particularly effective, gene therapy in somatic cells (i.e., insertion of a functional OAT gene into patients' cells) could be a reasonable therapeutic alternative for patients suffering from GA.

Vector construction.—The 1.4-kilobase (kb) EcoRI/HindIII fragment containing the entire human OAT cDNA was inserted into EcoRI/XhoI linearized retroviral vector LXSN to generate the recombinant vector pLXSN/OAT. All plasmids were grown in *Escherichia coli* strains HB 101 and DH 5 *Alfa*.

Cell lines.—The murine retroviral packaging cell line GP+E-86 was grown in Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum. We seeded ecotropic cells at 2×10^5 cells per 10 cm dish and transfected with 10 μg of undigested pLXSN/OAT plasmid DNA, using the calcium-phosphate method. Twenty-four hours later cells were washed twice with phosphate-buffered saline (PBS) and grown in the presence of 1 mg/ml of geneticin (G418) for 1 week in 24-well plates. Supernatants from different wells were harvested, filtered through 0.2 μm filters, and tested for viral titer on 14-day-old C57Bl/6 murine embryonal fibroblasts. After 1 day, the culture medium was replaced by G418 culture selection medium. Resistant clones were stained with methylene blue and counted.

OAT immunodetection.—Subconfluent 10-cm dishes of transduced and nontransduced murine embryonal fibroblast cells were grown for 16 hours in the culture conditions described above, then harvested and lysed in 500 mM sodium chloride (NaCl), 50 mM Tris (pH 7.5), 1% NP40. Protein extracts from the same number of transduced and nontransduced cells were subjected to preparative sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to nylon filters (Schleicher and Schuell). After saturation in PBS solution containing 5% nonfat dry milk, the strips were incubated with anti-OAT or with preimmune serum at the same dilution—1/100—for 1 hour at room temperature, then washed three times at 20°C for 20 minutes in 1% NP-40, 150 mM NaCl, and 50 mM Tris/HCl (pH 7.5). The strips were incubated with goal anti-IgG antibodies conjugated with peroxidase. Anti-antibodies were visualized by visiblot™ AP.

Major Findings

We have achieved the construction of Moloney murine leukemia virus (Mo-MuLV)-based recombinant retrovirus vectors expressing a human OAT cDNA under the control of a Mo-MuLV long-terminal repeat. Neomycin phosphotransferase was included as a selectable marker in these recombinant retroviruses. Murine embryonal primary fibroblasts, as well as embryonal stem cells, have been transduced with helper virus-free recombinant retrovirus particles generated from a GP+E-86 packaging cell line previously transfected with the described construct. We have successfully transduced several cell lines, as shown by Northern hybridization analysis

and immunodetection via rabbit polyclonal antibodies raised against human OAT.

Significance to Biomedical Research and the Program of the Institute

The improvement in visual function on reduction of ornithine accumulation suggests that the physiopathology of GA may involve (1) a direct toxic effect of ornithine; (2) a deleterious effect of metabolic alterations, occurring as a result of hyperornithinemia; or (3) both. However, despite many efforts, no therapy has been totally successful in treating this genetic disease.

It is possible to correct a genetic disease by directing the treatment to the site of the defect itself (i.e., "the mutated gene"), rather than against secondary or pleiotropic effects due to the mutant gene. A retrovirus-based delivery vehicle is likely to be the best choice for introducing a functional gene into somatic cells, thus achieving gene therapy of hereditary genetic diseases. Since the first successful retrovirus-mediated transfer of the adenosine deaminase gene into lymphocytes of patients suffering from a lethal immune deficiency, gene therapy has been viewed as a reasonable, feasible approach to treating human genetic diseases. Since then, work has focused on several other genes, such as those encoding low-density lipoprotein, factor IX, and the cystic fibrosis transmembrane conductance-regulator gene. Further research is under way, with the aim of clinical trial.

As shown by several groups, retroviral vectors can stably introduce genes into a variety of cultured cells. Defective retroviruses have been proposed as carriers to transfer functional genes to patients suffering from human genetic diseases.

To attempt gene transfer via somatic cell lines to patients suffering from genetic deficiency of OAT, we designed and made a Mo-MuLV-based retroviral vector carrying a functional human OAT gene. We analyzed its stable integration and expression in murine embryonal fibroblasts and embryonic stem cells. To test the production of OAT transcript from the recombinant retroviral virus present in the transduced cell lines, we performed Northern blot analysis, using a total RNA preparation from cell lines both transduced and nontransduced by OAT retrovirus. The results obtained show the production of significant amounts of mRNA transcript exclusively in the transduced cell lines that hybridize with an OAT cDNA probe. No reaction was detected in wild-type fibroblasts.

The absence of cross-reaction with murine OAT may be due to the high stringency during Northern blot washes or to the fact that mouse embryonal fibroblasts do not produce OAT enzyme. Moreover, when Western blot analysis of proteins extracted from the various cell lines used (including transduced mouse embryonal fibroblasts) was performed with rabbit polyclonal antibodies raised against two peptides of human OAT, we observed a specific reaction in only those extracts prepared from the cell lines transduced by the OAT provirus. The same polyclonal antibodies tested in Western blot against protein extracts from human retinal pigmented epithelium—fibroblasts and HeLa cell lines—showed a similar reaction on just one polypeptide of the same apparent molecular weight as that detected in the transduced fibroblasts. Taken together, these results reveal in transduced cells the expression of a single OAT transcript and a single OAT polypeptide.

We show here the ability to produce a retrovirus vector carrying and expressing a functional human cDNA coding for OAT, opening up the possibility of considering replacement gene therapy for OAT-deficient patients who suffer from GA disease.

Proposed Course

Our future efforts will focus on the following issues: (1) the enzymatic activity of OAT produced by genetically modified somatic cell lines, which we will further investigate via recombinant retrovirus; (2) assessment of the target cell types for gene product delivery; (3) investigation of the effect of overexpression of OAT in transgenic mice that express human OAT; and (4) characterization of the murine OAT genomic gene, prior to engineering the appropriate homologous recombination vector.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00232-08 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interferon System in Cellular Function and Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John J. Hooks	Ph.D.	Head, Section on Immunology and Virology	LI, NEI
Others:	Caroline Percopo	M.S.	Biologist	LI, NEI
	Chandrasekharam Nagineni	Ph.D.	Visiting Scientist	LI, NEI

COOPERATING UNITS *(if any)*

Department of Pathology, The George Washington University Medical Center (Barbara Detrick, Ph.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunology and Virology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cytokines, such as interferon-gamma (IFN-γ) and interleukin 2 (IL-2), are a group of specialized hormone-like proteins which exert profound influences on cellular development and on a variety of cellular functions. This project has concentrated on studying the ways in which cytokines interact with cells of the immune system and with cells in the ocular microenvironment. We have shown that IFN-γ and IL-2 are found within the inflamed eye in association with T-cell infiltration and major histocompatibility complex (MHC) class II antigen expression on infiltrating cells and on retinal pigment epithelium (RPE) cells. Furthermore, IFN-γ-activated RPE cells can process and present antigens to helper T lymphocytes.

Experimentally we demonstrated that isolated human RPE cells can be induced to produce another lymphokine, IL-6, following incubation with IFN-γ. IL-6 is a potent inflammatory cytokine capable of enhancing antibody production and cytotoxic T-cell activities. These studies indicate that cytokine-mediated activation of RPE cells may be a basic component of ocular immunity and an important aspect of RPE cell transplantation.

Retinoblastoma cells are an important model for exploring human malignancy and differentiation. Using these cells we showed that IFN-γ can regulate MHC class I genes at both transcriptional and posttranscriptional levels. In addition, this modulation is not associated with downregulation of N-myc oncogene expression.

These observations indicate that IFN-γ-induced MHC class I and class II antigen expression may serve as a local amplification system in autoimmune and inflammatory eye disease. A better understanding of the role of cytokines in the mechanisms involved in the development of autoimmunity and inflammation may be beneficial in developing treatments for these diseases.

Project Description

Objectives

This project is designed to determine the bioregulatory actions of interferon (IFN) and other cytokines and to evaluate their regulatory actions in the pathogenesis of disease.

Methods

We assayed human IFN, using inhibition of vesicular stomatitis virus plaque formation in human amnion (WISH) cells. IFNs were characterized by neutralization of antiviral activity with monoclonal anti-IFN immunoglobulin. Interleukin 2 (IL-2) biological activity was assayed by induction of proliferation of CTL cells. Interleukin 6 (IL-6) activity was assayed by an enzyme-linked immunosorbent assay, immunoblot assays, and Northern blots. Analytical flow cytometry was used to quantitate retinal proteins. Gene transcription techniques, such as Northern blot analysis and nuclear runoff transcription assays, are being used to evaluate IFN-γ modulation of retinal proteins.

Major Findings

IFN activation of retinal pigment epithelial (RPE) cells.—Numerous studies indicate that a variety of autoimmune diseases are associated with the IFN-γ-induced tissue-specific expression of major histocompatibility complex (MHC) class II molecules. During the past 5 years, we have identified various steps that may be involved in ocular immunopathologic mechanisms. In these studies of retinal degenerations and autoimmune diseases, we showed that a critical regulatory cell in the retina, the RPE cell, is capable of expressing MHC class II determinants. We also can detect IFN-γ *in situ* and in retinas from patients with inflammatory eye diseases, as well as in MHC class II-positive RPE cells. In addition, freshly isolated human RPE cells can express these determinants following treatment with IFN-γ.

In animal model systems, we found that inoculation of recombinant IFN-γ induces Ia expression of ocular cells, and treatment with anti-Ia antibodies can eliminate or inhibit experimental autoimmune uveitis. Most recently we showed that the RPE cell may be playing an important role in ocular immunity, acting as a resident antigen-presenting cell in the retina.

During the past year we have provided the most recent piece of experimental evidence implicating a role for cytokine-activated RPE cells in autoimmune phenomena by showing that the RPE cell is capable of producing the cytokine IL-6. RPE cell cultures were established from human donor eyes. These isolated RPE cells do not produce IL-6 alone; IFN-γ induces these cells to produce IL-6 in a dose-dependent manner. Moreover, IFN-γ can synergize with tumor necrosis factor (TNF) to produce IL-6 in human RPE cells. IL-6 is a potent cytokine which can act on B lymphocytes to induce growth and antibody production. It can also act on T lymphocytes to induce IL-2 production, IL-2 receptor expression, and cell proliferation. These studies further substantiate the concept that cytokine-mediated activation of RPE cells may be a basic component of ocular immunity and may have major immunological consequences for RPE cell transplantation studies.

Cytokine-induced modulation of cellular proteins in the retina and retinoblastoma.—Retinoblastoma cells are an important model for exploring human malignancy and differentiation. These multipotent embryonic cells are capable of differentiating into neuronal, glial-like, and RPE-like elements. We have shown that flow cytometric analysis can be used to measure the expression of both cytoplasmic and cell surface proteins in retinoblastoma cells. We used this technique to monitor changes in the expression of selected cellular proteins after exposure to specific cytokines and found that MHC class I molecules were augmented by IFN-α and IFN-γ but not by TNF. However, the MHC class II molecules were augmented by IFN-γ but not by IFN-α or TNF. The neuronal markers IRBP and PR-6, the glial-like marker GFAP, and the RPE cell markers RPE-9 and RPE-15 were not altered by any of the cytokines tested.

The mechanism of induction of MHC class I and II antigens by IFN in retinoblastomas is not known. We therefore have initiated studies to compare IFN-α, IFN-β, and IFN-γ in their ability to induce the expression of class I antigens and to investigate the role of transcriptional and posttranscriptional mechanisms in this induction. We found that IFN-γ increased HLA class I antigen expression and induced a fivefold increase in its transcription rate. Posttranscriptionally IFN-β and IFN-γ increased

steady-state mRNA for the HLA-B7 gene. These effects were not associated with down-regulation of N-myc oncogene nuclear transcription. Moreover, dexamethasone did not affect the IFN-γ-induced expression of HLA class I molecules. These studies implicated both transcriptional and posttranscriptional mechanisms in the modulation of class I molecule expression by IFNs.

Significance to Biomedical Research and the Program of the Institute

These studies highlight the fact that the release of cytokines, such as IFN-γ, within the ocular microenvironment and the subsequent induction of cytokines and MHC class I and II antigen expression on resident and infiltrating cells may be critical elements in a cascade leading to ocular cell destruction. The retinal cell that may play a critical role in autoimmune uveitis is the RPE cell. IFN-γ-induced activation of RPE cells may participate in autoimmune disease in the ocular microenvironment.

Cytokines produced and localized in the eye may play critical roles in normal physiology, pathogenic mechanisms, and therapeutic approaches. Since the RPE cell is a pivotal regulatory cell in the retina, an understanding of how cytokines interact with this cell will shed light on avenues for therapeutic intervention in pathogenic states and in transplantation.

Proposed Course

We plan to continue our evaluation of the role of cytokines in autoimmunity and inflammation. We are now developing systems in rat models to monitor directly the effects of altering cytokine production on inflammatory eye diseases. Moreover, we will continue to characterize the antigen-presenting ability of the RPE cell in a variety of antigens and viruses.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

Barez S, Boumpas D, Percopo C, Anastassiou ED, Hooks JJ, Detrick B: Modulation of major histocompatibility complex (MHC) class I genes in human retinoblastoma cells by interferons: Evidence for both transcriptional and post-transcriptional regulation. *Invest Ophthalmol Vis Sci* 34:2613-2621, 1993.

Detrick B, Hooks JJ: Cytokines and effector molecules in human immunology, in Leffell MS, Bias WB, Donnenberg AD, Rose NR (eds): *CRC Handbook of Human Immunology*. Boca Raton, CRC Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00233-08 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on the Bioregulatory Aspects of the Retinal Pigment Epithelial Cell

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John J. Hooks	Ph.D.	Head, Section on Immunology and Virology	LI, NEI
Others:	Chandrasekharam Nagineni	Ph.D.	Visiting Scientist	LI, NEI
	Caroline Percopo	M.S.	Biologist	LI, NEI
	T. Michael Redmond	Ph.D.	Research Biologist	LRCMB, NEI
	David Parks	M.D.		LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI

COOPERATING UNITS *(if any)*

Hôpital St. Louis, Paris, France (Lawrence Boumsell, M.D.); University of Nice, France (Alain Bernard, M.D.); National Institute of Dental Research (Reuben Siraganian, M.D.); The Johns Hopkins University (Stanley A. Vinores, Ph.D.; Peter Campochiaro, M.D.); Department of Pathology, The George Washington University Medical Center (Barbara Detrick, Ph.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunology and Virology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.9	0.5	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The retinal pigment epithelial (RPE) cell plays a basic role in maintaining the structural and physiological integrity of the neural retina. We have isolated and propagated RPE cells *in vitro* and have developed monoclonal antibodies directed against human RPE cells. We are using these techniques and reagents to evaluate molecular, biochemical, and biological properties of the RPE cells.

Since the monoclonal antibodies detect epitopes present solely on RPE cells, they provide a unique opportunity to evaluate a variety of aspects of RPE cell development and function. Studies on RPE cell development indicate that the epitopes appear only after the cells have begun terminal differentiation. Moreover, studies on RPE migration demonstrate the value of these antibodies in evaluating epiretinal membrane formation. The RPE epitope is a 67-kD protein that is closely associated with the microsomal membrane. A cDNA clone that has been isolated codes for a protein which does not match any other sequences in the data bases. Studies are in progress to propagate and transplant RPE cells in various animals. We have propagated human RPE cells *in vitro* and evaluated their ability to respond to cytokine activation.

RPE cells respond to retinal aberrations by dying, proliferating, migrating, losing phagocytic function, expressing major histocompatibility complex (MHC) class II antigens, and presenting antigens to T lymphocytes. The techniques and reagents obtained in these studies allow us to evaluate the mechanisms involved in aberrant responses of RPE cells. Moreover, they provide a framework for evaluating RPE cell transplantation.

Project Description

Objectives

The aim of this project is to evaluate the molecular, biochemical, and various biologic properties of retinal pigment epithelial (RPE) cells in normal and disease states. Moreover, we are evaluating RPE cell transplantation.

Methods

RPE cells are isolated and propagated *in vitro*. Monoclonal antibodies are generated in mice by fusing mouse spleen cells with myeloma cells. Antibodies to the RPE cells are evaluated by immunoperoxidase and Western blot assays. The effects of drugs and cytokines are evaluated by cell viability and proliferation assays and by nuclear transcription runoff assays.

Major Findings

Evaluation of epitopes identified by monoclonal antibodies.—We have identified two mouse IgG3 monoclonal antibodies that react with RPE cells from a variety of species, ranging from man to frog. Since these antibodies detect epitopes present solely on RPE cells, they provide us with the unique opportunity to evaluate a variety of aspects of RPE cell development and function.

Electron microscopic immunocytochemistry revealed labeling patterns for the two RPE antibodies that are very similar. In human eyes, staining was localized to surface and intracellular membranes and the cytoplasm. Staining occurred predominantly on the apical surface of the RPE cells. The RPE protein, a 65-kD protein, was isolated by polyacrylamide gel electrophoresis and transferred to nitrocellulose blot, and the sequence of its amino acid residues was determined. The amino acid sequence was used to design a synthetic cDNA probe. A bovine cDNA library was screened, and cDNA clones were isolated and characterized. The cDNA insert is 3,115 base pairs; the open reading frame encodes a protein of 533 amino acids. The RPE protein does not match any other sequence in the data bases. The protein expressed in *Escherichia coli* has a molecular weight similar to that of the native protein. In addition, we used Northern blotting with the cDNA to detect protein mRNA in RPE cells.

In studies of the developmental expression of RPE and photoreceptor determinants in the rat retina, we had previously shown that the expression of these determinants in rats is absent the day of birth and detectable at postnatal Day 6. Recent studies show that RPE cells express their determinants shortly before the first outer segments are detected and that the posterior-anterior progression of outer segment formation matches a similar progression of the expression of the RPE determinants. These data indicate that the RPE resumes its maturation during the first postnatal week and that RPE maturation and outer segment growth can be correlated.

RPE cell transplantation.—Recent studies indicate that RPE cell transplantation may be beneficial in restoration of the retinal architecture in selected retinal degenerations. It is essential to develop methods for large-scale preparations of RPE cell cultures for somatic cell genetic engineering manipulation. We are in the process of evaluating various parameters for human and rat RPE cell culture and transplantation. Preliminary studies show that we can successfully inoculate human RPE cells into the rat retina. Evaluation of the immunologic parameters of this transplantation process is under way.

Significance to Biomedical Research and the Program of the Institute

The monoclonal antibodies developed in this study are the first directed solely at the RPE cell. These antibodies are potentially useful in identifying RPE cells *in situ* and *in vitro*. These antibodies, which can be used to monitor RPE cellular functions, may provide a better understanding of the role of RPE cells in retinal degenerative disorders. Identification of the cDNA and proteins detected by the monoclonal antibodies may provide the framework on which to evaluate specific RPE cell functions. RPE cell transplantation to correct retinal degenerative processes is being actively investigated in a number of laboratories. The studies reported here provide the basis for evaluation of RPE cell transplantation.

Proposed Course

1. We will continue to characterize these antibodies and their effects on cell function *in vivo* and *in vitro*.

2. We will isolate, propagate, and characterize RPE cells for transplantation studies in animals and

humans. We will design effective ways to maintain the cell in culture and to measure and monitor cell function.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

Hamel CP, Tsilou E, Harris E, Pfeffer BA, Hooks JJ, Detrick B, Redmond TM: A developmentally regulated microsomal protein specific for the pigment epithelium of the vertebrate retina. *J Neurosci Res* 34:414-425, 1993.

Hamel CP, Tsilou E, Pfeffer BA, Hooks JJ, Detrick B, Redmond TM: Molecular cloning and expression of RPE65, a novel retinal pigment epithelium-specific microsomal protein that is post-transcriptionally regulated *in vitro*. *J Biol Chem* 268:15751-15757, 1993.

Vinores SA, Orman W, Hooks JJ, Detrick B, Campochiaro PA: Ultrastructural localization of RPE-associated epitopes recognized by monoclonal antibodies in human RPE and their induction in human fibroblasts by vitreous. *Graefe's Arch Clin Exp Ophthalmol* 231:395-400, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00240-07 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Virus Infections in the Eye

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John J. Hooks	Ph.D.	Head, Section on Immunology and Virology	LI, NEI
Others:	Caroline Percopo	M.S.	Biologist	LI, NEI
	Yun Wang	M.D.	Guest Worker	LI, NEI
	Miguel Burnier	M.D.	Visiting Scientist	LI, NEI
	Ingeborg Kirch	M.D.	Guest Worker	LI, NEI
	Yusuke Komuraski	M.D.	Guest Worker	LI, NEI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunology and Virology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our studies of various virologic and immunopathologic processes that occur when viruses replicate in the ocular microenvironment comprise four areas: (1) coronavirus infection in ocular and optic nerve cells, (2) the possible roles of viruses in human diseases, (3) antiviral therapeutic actions of cytokines and drugs, and (4) molecular diagnosis and pathogenesis of cytomegalovirus (CMV) infections in man. We have established that murine coronavirus can induce ocular disease and may be used as a model system for studying retinal degenerative diseases. This model has many unique features. The virus is capable of inducing an acute infection in the presence of mild retinal vascular inflammation. Initial retinal damage is followed by clearance of the virus and progressive retinal degeneration, even months after the virus is gone. The virus replicates predominantly in Müller cells and also can be detected in retinal pigment epithelium (RPE) and photoreceptor cells. Recent studies show that there are genetic and immunologic components to this disease. The retinal degenerative pathologic manifestations of the disease can be influenced by the genetics of the host, i.e., some strains of mice are resistant to virus-induced retinal degenerative changes. The pathologic changes also are closely related to the development of antiretinal and anti-RPE antibodies. These findings suggest a role for autoimmunity in the pathogenesis. This disease may be considered a model for degenerative diseases of the pigment epithelium and photoreceptors in humans.

The need for effective drug treatment and prevention of herpes virus and other viral diseases has assumed growing importance. We found that leukoregulin, a naturally occurring immunologic cytokine, not only increases the antiviral actions of the drug acyclovir but also directly inhibits herpes simplex virus replication, demonstrating that combination immunotherapy and chemotherapy can produce substantial inhibition of herpes virus replication and providing a rationale for applying this approach to treating virus infections.

Studies initiated this past year indicate that CMV is capable of replicating within human RPE cells *in vitro*; however, replication is limited at the level of immediate early protein production. The low frequency of expression of immediate early viral proteins in RPE cells and the subsequent slow replication of CMV may be critical variables in terms of their relationship to viral persistence and activation within the retina.

Project Description

Objectives

This project was designed to determine the various effects of virus infections on the ocular microenvironment and to study modes of antiviral therapy.

Methods

This study involves the propagation and quantitation of viruses, such as herpes simplex virus type 1 (HSV-1), coronaviruses, and cytomegalovirus (CMV), both *in vitro* and *in vivo*. It also includes immunocytochemical analysis of infected cells and tissues. Techniques used in characterization of virus infection include flow cytometric analysis, Western blot analysis, Northern blot analysis of HSV thymidine kinase, *in situ* hybridization, and amplification of viral genes by polymerase chain reaction (PCR). Techniques used in characterization of antivirus antibodies include enzyme-linked immunosorbent assay and neutralization assays.

Major Findings

Coronavirus infection in the eye.—The murine coronavirus, mouse hepatitis virus (MHV), JHM strain, induces a retinal degenerative disease in adult Balb/c mice. The disease consists of an acute phase lasting from 2 to 8 days, during which virus is detected within the retina and initial pathology is noted in the retinal pigment epithelium (RPE) and photoreceptor layers. This is followed by a late phase lasting from 1 to 14 weeks, during which virus is not detected but retinal degenerative changes continue, with reduction of the photoreceptor layer, loss of interphotoreceptor retinoid-binding protein, and retinal detachments. This model provides evidence that viruses can trigger retinal degenerative processes and may offer insight into pathogenic mechanisms in retinal degenerative diseases of humans. During the past year we have evaluated three aspects of this disease process: (1) immunologic aspects of the disease, (2) genetic predisposition to the disease, and (3) use of electroretinography (ERG) to monitor the disease process.

In the coronavirus-induced retinopathy, the late phase of the JHM-induced disease was associated with the lack of direct evidence for viral replication within the retina. This observation suggested that the continued degenerative process may be associated with alterations directly induced by virus replication during the first few days after infection, or it may be associated with additional factors. Because viruses are known to trigger an autoimmune phenomenon and some human retinopathies may be associated with autoantibody formation, we studied the possible production of antiretinal autoantibodies. We found that the retinal degenerative process is associated with the presence of antiretinal autoantibodies. In total, 22 of 23 sera samples collected from 10 to 70 days after JHM virus inoculation of Balb/c mice contained antiretinal autoantibodies. These autoantibodies were not found in sera from normal or mock-injected mice.

Antibodies to retinal tissue were identified by two distinct patterns of immunoperoxidase staining on frozen sections of normal rat eyes—retinal autoantibodies and RPE autoantibodies. The antiretinal autoantibodies first appeared as IgM class antibodies which shifted to IgG class autoantibodies. The anti-RPE cell autoantibodies were predominantly of the IgG class. Sera positive for these autoantibodies did not stain with liver or kidney sections, but two of three did react with rat brain sections.

We also evaluated a second mouse strain, CD-1, because these animals respond to JHM virus inoculation by developing only the early phase of this disease, i.e., vasculitis. On Day 10 postinoculation (pi) the retinal architecture had a normal appearance. In these mice, which are free of a retinal degeneration, antiretinal autoantibodies are not produced. However, as noted in the Balb/c mice, antivirus neutralizing antibodies were produced in the infected CD-1 mice. These findings suggest a role for autoimmunity in the pathogenesis of murine coronavirus-induced retinal degeneration.

Since the genetic composition of the host and the virus can determine the response to infection and the resulting pathology, we evaluated the effect of MHV infections on different strains of mice. The JHM and A59 strains of MHV were propagated in rat L2 cells. Balb/c, CD-1, and A/J mice were inoculated by the intravitreal route with $10^{4.5}$ $TCID_{50}$/0.5 µl of virus or with uninfected tissue culture preparations (mock injection). At various times after infection, the eyes were removed and evaluated histologically, and sera were assayed for the presence of virus-neutralizing antibody. Both JHM and A59 strains of MHV induced similar retinal diseases.

We observed two distinct phases of coronavirus-induced retinopathy, retinal vasculitis (Days 3-6) and retinal degeneration (Days 10-20), in Balb/c mice. In contrast, we saw only the early stage of disease in CD-1 mice. We observed typical retinal vasculitis at 3-6 days pi. However, by Day 10 pi the retinal architecture had returned to a normal appearance. No retinal degeneration was observed. The third strain, A/J mice, displayed a biphasic disease but with a mild degenerative component. All strains of mice responded to the retinopathy by developing antivirus-neutralizing antibody at similar levels. These studies demonstrate that the pathologic manifestations of a virus infection in the retina can be influenced by the genetics of the host.

The third phase of these studies incorporated ERG evaluation of the development of the virus-induced retinopathy. At various times after inoculation, animals were dark-adapted for 60 minutes and anesthetized, after which ERGs were recorded between a wick electrode touching the cornea and a needle electrode placed subcutaneously at the forehead. Light stimuli were provided by a xenon arc light source and focused onto the eye via a fiberoptic bundle. We varied the light intensity via neutral-density filters. Five responses were averaged for each light intensity.

On Day 3 pi all virus-infected mice had slightly depressed ERGs and retinal vasculitis was seen. In contrast, on Days 8 and 20 pi all these mice had subnormal or abolished responses, and retinal degenerative changes were clearly apparent. On Day 10 pi 57% of the mice (4/7) had abolished responses and 43% (3/7) had subnormal responses. On Day 22 pi 50% (4/8) had abolished responses, while the remaining 50% had subnormal ERGs. Mice inoculated with virus-free tissue culture preparations had minor alterations in their ERG patterns. However, at Day 20 pi these mock-injected mice displayed ERG patterns similar to those of normal, uninfected mice. ERG studies in this murine model provide an indirect but objective means of measuring visual function, serving as the basis for future studies of treatment effects.

In summary, this model is characterized by the replication of JHM virus in the retina, producing an acute necrotizing disease of the sensory retina that results in only a mild inflammatory response and a long-lasting disease (>14 weeks). In these studies we identified a progressive degenerative disease in the retina that may be initiated by an acute virus infection in the absence of major inflammatory response. During the past year these studies have clearly indicated that this retinal degenerative process has viral, immune, and genetic components. How the genetic and immunologic factors interact to influence the development of retinal degenerations is the intriguing aspect of this model.

Possible role of viruses in human eye diseases.—We have initiated studies to evaluate the possible involvement of viruses in the pathogenic processes of a variety of human eye diseases. We are now collecting serum samples and ocular tissue in order to use seroepidemiologic approaches for the detection of virus and viral antigens via immunocytochemical staining, *in situ* hybridization, and PCR assays.

Antiviral therapeutic actions of cytokines and drugs.—The need for effective treatment and prevention of herpesvirus and other viral diseases has assumed growing importance during the past 10 years. The development of targeted antiviral agents through combination therapy is becoming an important strategy. One strategy consists of the development of cytokines or lymphokines in combination with chemotherapy to treat malignancy and infections. Using this approach, we recently showed that the cytokine leukoregulin could enhance the anti-HSV actions of acyclovir (ACV).

Cytokines are a group of specialized hormone-like proteins that can exert profound influences on cellular development and a variety of cellular functions. As a lymphokine that performs unique regulatory activities in transformed cells, the leukoregulin molecule, which is produced by a variety of lymphoid cells, is a multifunctional cytokine. It can prevent chemical carcinogen transformation, inhibit neoplastic cell proliferation, and augment target cell sensitivity to natural killer cell cytotoxicity. Furthermore, this cytokine has been shown to increase membrane permeability of tumor cells and to increase drug uptake in these cells. We recently showed that leukoregulin can selectively increase membrane permeability in HSV-1-infected cells but not in normal (i.e., uninfected) cells.

In addition, we have recently shown that leukoregulin enhances the anti-HSV actions of ACV. The cells were exposed to the cytokine and/or ACV for only 3 hours early in the replication cycle. Because the continued presence of ACV greatly enhances antiviral activity, we evaluated the effect of the

continuous presence of leukoregulin on HSV replication. Human amnion epithelial (WISH) cells were infected with HSV-1 (Wendy and F strains) and vesicular stomatitis virus (VSV). Following a 90-minute incubation period, we washed the cells and treated them with media, leukoregulin, ACV, or leukoregulin plus ACV. We evaluated virus replication by plaque assays while testing virus and cellular protein expression by immunoblotting. The continuous presence of leukoregulin (0.1 unit) inhibited HSV-1 plaque formation by 50-80% in the Wendy and F strains, respectively. In contrast, leukoregulin did not affect VSV replication. Immunoblot analysis revealed that the expression of the 89-kD HSV-1 protein was inhibited by 50%, whereas the cellular protein, actin, was not affected by leukoregulin treatment. Moreover, leukoregulin treatment did not alter the ability of the cells to incorporate tritiated thymidine.

Initial evaluations of the effect of leukoregulin on HSV transcription indicated that the cytokine did not alter the level of expression of HSV tk mRNA. These studies show that leukoregulin not only enhances the antiviral actions of ACV but also can act to inhibit HSV-1 replication directly. These findings, which demonstrate that combination immunotherapy (cytokines) and chemotherapy can substantially inhibit herpesvirus replication, provide rationale for the application of this approach to the intervention of virus infections.

CMV replication within the retina.—CMV infections are frequent complications in kidney and bone marrow transplant patients and HIV (human immunodeficiency virus) patients. Because the mechanisms by which CMV is activated and replicates within the retina are not known, we evaluated the ability of human CMV to initiate replication in human RPE cells and compared the results with finding in studies of human fibroblasts (HEL) and WISH cells. Human RPE cells obtained from donor eyes were propagated *in vitro* and infected at an input multiplicity of 1. CMV replication was evaluated in three ways: (1) detection of viral antigen by immunofluorescence and flow cytometry, (2) detection of virus-induced cpe, and (3) assaying for infectious virus.

We found no evidence of viral replication in the WISH cells. In contrast, CMV replication was detected in both RPE and HEL cells. In HEL cells, IE, E, and L proteins were detected at 5, 48, and 48 hours, respectively. In RPE cells these proteins were detected somewhat later—at 24, 48, and 72 hours. We noted a striking difference in the percentage of cells expressing IE protein: After 72 hours, 100% of the HEL cells expressed IE protein, whereas after 7 days, less than 1% of the RPE cells expressed this protein. Analysis of the production of infectious virus revealed that viral infectivity and cpe were maximal at Day 5 in HEL cells and at Day 30 in RPE cells.

This study demonstrates that, although all of the RPE cells were capable of becoming infected with CMV, less than 1% of the cells expressed IE viral proteins early in the infection cycle. The low frequency of expression of IE viral protein in RPE cells and the subsequent slow replication of CMV may be critical variables in terms of their relationship to viral persistence and activation within the retina.

Significance to Biomedical Research and the Program of the Institute

Elucidating the factors involved in viral spread and pathogenesis will yield a better understanding of diseases of viral etiology. We have established a new virus model for retinal degenerative processes in adult animals. This model has many unique features. It is capable of inducing acute infection in the presence of mild retinal vascular inflammation. The initial retinal damage is followed by clearance of the virus and progressive retinal destruction, even months after the virus is gone. Moreover, development of the retinal degenerative process is determined by the genetics of the host; it involves the development of antiretinal autoantibodies. This model should assist us in understanding the pathogenesis of selected human diseases of unknown etiology.

We have identified a cytokine, leukoregulin, that selectively increases membrane permeability in virus-infected cells. We also have shown that combined cytokine and drug therapy can produce substantial inhibition of HSV replication. Moreover, the continuous presence of the cytokine can directly inhibit virus replication. The data from these studies provide rationale for the application of this approach to the interventive treatment of virus infections.

We have shown that CMV replicates within RPE cells in a slow, limited manner. Evaluation of the molecular aspects of this defect may provide critical clues in terms of the virus' ability to establish

persistent infections and the factors initiating viral activation within the retina.

Proposed Course

1. We will continue to evaluate coronavirus infections of the eye. The role(s) of genetic factors and autoantibodies in the pathogenesis of retinal degenerations will be evaluated. The data obtained will be correlated with what is known about human retinal degenerative disorders.

2. We will initiate studies to determine whether certain viruses can replicate in retinal tissues and cells. Infected cells will be evaluated for the release or expression of uveitogenic proteins.

3. We will continue to collect samples and initiate studies to detect the involvement of viruses in human eye diseases.

4. We will continue to evaluate combinations of leukoregulin and chemotherapeutic agents for the management of virus infections.

5. We will evaluate the molecular diagnosis and pathogenesis of CMV infections in the eye.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

Burnier M, Wang Y, Detrick B, Hooks JJ: Retinal manifestations of a murine coronavirus infection: A histopathological and ultrastructural study. *Exp Pathol*, in press.

Hooks JJ: Ocular virology, in Tabbara K (ed): *Infections of the Eye.* Boston, Little, Brown & Co, in press.

Hooks JJ, Percopo C, Wang Y, Detrick B: Retina and retinal pigment epithelial cell autoantibodies are produced during murine coronavirus retinopathy. *J Immunol* 151:3381-3389, 1993.

Wang Y, Detrick B, Hooks JJ: Coronavirus replication within the retina: Analysis of cell tropism in mouse retinal cell cultures. *Virology* 193:124-137, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00287-01 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Toxoplasmosis Infections in the Eye

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John J. Hooks	Ph.D.	Head, Section on Immunology and Virology	LI, NEI
Others:	M. Cristina Martins	M.D.	Guest Worker	LI, NEI
	Chandrasekharam Nagineni	Ph.D.	Visiting Scientist	LI, NEI
	Miguel Burnier	M.D.	Visiting Scientist	LI, NEI
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Toxoplasma gondii infections are a major source of visual loss and blindness. Ocular toxoplasmosis may occur as a result of congenital or acquired infections and as a manifestation of immunosuppression, particularly as a result of transplantation or AIDS (acquired immune deficiency syndrome). Due to the recent resurgence of acquired ocular toxoplasmosis in Brazil and the worldwide complications of toxoplasmosis in HIV (human immunodeficiency virus) infections, we initiated studies to develop a model of acquired toxoplasmosis to evaluate the molecular mechanisms of pathogenesis and therapeutic strategies.

We have developed an animal (murine) model of ocular toxoplasmosis that is characterized by retinal inflammation, chorioretinal scarring, retinal disorganization, and cyst formation. Retinal disease occurs in three different strains of mice following inoculation with toxoplasmosis by the subcutaneous or intraperitoneal routes. This model of acquired ocular toxoplasmosis is being used to evaluate the efficacy of new antiparasitic agents in controlling the development of retinal cyst formation and retinal inflammation.

Project Description

Objectives

This project was designed to develop an animal model of acquired ocular toxoplasmosis, which will be used to evaluate molecular mechanisms of ocular pathogenesis and to evaluate new antiparasitic drugs and cytokines.

Methods

This study involves the propagation and quantitation of *Toxoplasmosis gondii* strains *in vitro* and *in vivo*, as well as immunocytochemical analysis of infected cells and tissues. Techniques used in characterization of *T. gondii* infections include histopathology, immunocytochemistry, *in situ* hybridization, and Western blot analysis. Techniques used in characterization of antitoxoplasmosis antibodies include enzyme-linked immunosorbent assay.

Major Findings

Adult Swiss Webster, C57BL6, and Balb/c mice were inoculated by the subcutaneous route or intraperitoneal route with 10 *T. gondii* cysts (S2C9 or ME49 strains) in a 1-ml volume. At various times after inoculation (i.e., Days 7, 14, 21, 28, and 42), we sacrificed the mice and removed and fixed the eyes and brains in 10% buffered formalin. Fifteen hematoxylin and eosin-stained sections of brain and eye were evaluated for the presence of *T. gondii* cysts.

By Day 14, 100% of the mice had developed cysts in the brain. Retinal inflammation also was noted in 100% of the animals by Day 14, and chorioretinal scars were observed in mice inoculated with both strains of *T. gondii*. Retinal cysts were found in mice 28 and 42 days after inoculation with the ME49 strain and 14 and 42 days after inoculation with the S2C9 strain in Swiss Webster mice. *T. gondii* cysts in the retina were detected in C57BL6 mice at 14, 21, 28, and 42 days after inoculation with the S2C9 strain. This study has identified an animal model of ocular toxoplasmosis characterized by retinal inflammation, chorioretinal scarring, retinal disorganization, and cyst formation.

Significance to Biomedical Research and the Program of the Institute

This is the first model of acquired toxoplasmosis that consists of retinal inflammation, degeneration, and parasitic cyst formation. It will allow us to evaluate the efficacy of new antiparasitic drugs in controlling the development of retinal cyst formation and retinal inflammation and scarring.

Proposed Course

We will evaluate drugs and cytokines in the control of the ocular manifestations of acquired toxoplasmosis infections.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00277-02 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Retinal Pigment Epithelium in Retinal Disorders

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The retinal pigment epithelium (RPE) plays a critical role in the regulation of retinal and choroidal function in normal and disease states. Due to limited availability of human tissues, an *in vitro* cell culture system is desired. Therefore we have developed and characterized the primary cell lines of human RPE from donor eyes obtained from eye banks. Using human RPE cell cultures as a model, we conducted investigations to examine the various roles of RPE in the pathophysiology of retinal disorders.

Human RPE cultures exposed to bacterial lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF-α), and interleukins 1 alpha and 1 beta (IL-1α, IL-1β) secreted large amounts of interleukin 6 (IL-6). Immunoblot and Northern blot analysis confirmed the presence of posttranslationally modified IL-6 protein and mRNA levels, respectively. Interferon-gamma (IFN-γ) acted synergistically with other mediators to stimulate 3- to 5-fold increases in IL-6 secretion. We extended our studies to examine the expression and secretion of intercellular adhesion molecule-1 (ICAM-1), a cell surface ligand for lymphocyte function-associated antigen-1 (LFA-1) expressed during inflammatory reactions, by human RPE. IFN-γ, TNF-α, and IL-1 increased significantly both cell surface expression (detected by immunofluorescence staining) and secretion into the medium (detected by ELISA). Secretion (shedding) of ICAM-1 by RPE cells in the presence of a mixture of IFN-γ (100 u/ml), TNF-α (1 ng/ml), and IL-1 (1 ng/ml) was cumulative, suggesting that combining these cytokines results in potent inflammatory reactions. The response of RPE cells to inflammatory mediators was rapid and sustained in the presence of stimulants but reversed to control levels quickly upon withdrawal, suggesting the reversibility of the responses of RPE to inflammatory signals. The effects of transforming growth factor beta (TGF-β) on RPE functions at cellular and molecular levels also are being studied. TGF-β increased the expression of heme oxygenase-1, an enzyme reaction that generates the antioxidant bilirubin, which helps in cellular defense mechanisms against oxidative stress.

The results clearly show that human RPE cells respond to specific inflammatory signals or infections by increased cellular expression and secretion of IL-6 and ICAM-1, which may in turn perpetuate immune reactions in the pathogenesis and/or prevention of retinal and choroidal diseases.

Project Description

Objectives

Human retinal pigment epithelial (RPE) cell cultures have been established from human donor eyes. Primary cell lines of RPE are used as an *in vitro* model to study the effects of growth factors, inflammatory mediators, and toxic compounds on biochemical, cellular, and molecular aspects of RPE structure and function. The usefulness of RPE cultures also are being evaluated for transplantation to restore retinal function in hereditary and age-related disorders.

Methods

Primary cell cultures are prepared by initial seeding of either freshly isolated RPE cells or RPE-choroid explants. Cells are grown in minimum essential medium supplemented with 10% fetal calf serum, nonessential amino acids, and antibiotics. We are attempting to develop serum-free hormonally defined medium to render cultured cells more suitable for transplantation therapy with minimal immune-related complications and consequent graft rejection.

Techniques required for cell culture, immunofluorescence, cytokine, and ICAM-1 assays by enzyme-linked immunosorbent assay (ELISA), gel electrophoresis, and Western and Northern blotting for proteins and RNA are developed and standardized in our laboratory to carry out these studies.

Major Findings

In the past, the age of the donor was considered very critical in preparing human RPE cultures because eyes from donors over 50 years did not yield fruitful cell lines, probably due to senescence-associated loss of viability. In these experiments, RPE cells were first dissociated from the eye cups by digestion with proteolytic enzymes, treatment that might have caused initial contamination with nonepithelial cells, from which it is impossible to purify epithelial cells. Therefore, we modified this method, using RPE-choroid explants, native and without harsh enzyme treatment, to initiate cell growth. Then, by careful monitoring of clusters of cells growing around the explants, we were able (on the basis of morphology combined with experience) to select purely epithelial cells and discard nonepithelial cells at the primary culture stage.

Using this technique, we established primary cell lines of human RPE from eyes obtained from 81- and 87-year-old donors. The epithelial nature of these cell lines was confirmed by immunochemical staining with monoclonal antibodies to cytokeratin. All of the cells expressed cytokeratin at different passages (3 to 10). Immunoblotting analysis of cellular proteins indicated cytokeratin 18 as the predominant cytokeratin in these cells. Because RPE is the only epithelial cell in the posterior segment (choroid-RPE-retina), these results establish without doubt that the cell lines developed are, in fact, RPE. The feasibility of using donor eyes from a population over 70 years of age for preparing RPE cultures is demonstrated.

Human RPE cultures secrete large quantities of inflammatory cytokine, interleukin 6 (IL-6), when exposed to inflammatory mediators—lipopolysaccaride (LPS), tumor necrosis factor alpha (TNF-α), IL-1α, and IL-1β. Western blot analysis revealed posttranscriptionally processed forms of IL-6 in the secreted proteins. Although interferon-γ (IFN-γ) induced the lowest levels of IL-6 by itself, it acted synergistically with other cytokines to stimulate threefold to fivefold increases in IL-6 secretion by RPE. Analysis of IL-6 secretion by ELISA and the expression of IL-6 mRNA by Northern blotting indicated rapid, sustained RPE responses to inflammatory mediators that can be reversed quickly upon withdrawal of the stimulus. Cell surface expression and secretion (shedding) of intercellular adhesion molecule 1 (ICAM-1), a cell surface glycoprotein ligand for lymphocytes, by RPE was significantly stimulated by inflammatory cytokines—IFN-γ, TNF-α, and IL-1. The presence of all these cytokines together appears to induce more potent secretion of ICAM-1. Regulation of the expression of ICAM-1 in RPE is under investigation through the use of monoclonal antibodies and cDNA probes.

Our observations suggest that, in response to the presence of the inflammatory cytokines produced by macrophages and lymphocytes that, for example, infiltrate the eye during inflammation caused by infection or autoimmune disease, RPE can locally produce IL-6 and ICAM-1. In turn, IL-6 aids in the proliferation and differentiation of lymphoid cells to regulate immunological phenomena. Secreted or cell surface ICAM-1 expressed on RPE helps in homing and concentration of lymphocytes near the sites of inflammation for immunoregulation.

Elevated levels of intravitreal IL-6, IL-1, TNF-α, and IFN-γ were reported in proliferative and other noncomplicated retinal detachments. Moreover, intravitreal injection of IL-1, TNF-α, or IL-6 induced uveitis in experimental animal models. These studies implicate local but not systemic increase in these cytokines as initiators of uveitis. Increases in the circulating ICAM-1 levels in the serum of uveitis patients and expression of ICAM-1 in epiretinal membranes in proliferative vitreoretinopathy, proliferative diabetic retinopathy, and macular pucker were reported, suggesting involvement of ICAM-1 in various diseases. Our studies indicate that RPE, possibly in association with other resident cells, reacts to inflammatory stimuli and participates in the immunopathologic mechanisms by secreting IL-6 and ICAM-1.

Basic fibroblastic growth factor (bFGF) and transforming growth factor beta (TGF-β) secreted by RPE are known to have both autocrine and paracrine actions on retina and choroid. bFGF and TGF-β are involved in various biological processes, such as cell proliferation, differentiation, wound healing, immunosuppression, and apoptosis. The roles of bFGF and TGF-β in RPE functions and the regulation of secretion of these growth factors by RPE are being investigated. We have studied the expression of heme oxygenase-1 (HO-1), an enzyme known to respond to oxidative stress, heat shock, heavy metals, and inflammatory agents, that offer protection against oxidative damage. Among ocular tissues, RPE has the highest activity of HO-1. Using specific polyclonal antibodies and PCR-generated probes, we have demonstrated that TGF-β increases HO-1 levels by fourfold to fivefold in human RPE cells within 4 hours. Toxic compounds—cadmium, lead, mercury, arsenite, and iodoacetate—are the most potent inducers of HO-1 in RPE. HO-1 catalyzes the oxidation of heme into biliverdin and carbon monoxide. Biliverdin is converted by nonlimiting enzymatic reaction into bilirubin, an antioxidant that offers cells protection against heat and oxidative stress.

Significance to Biomedical Research and the Program of the Institute

Primary cell lines of human RPE are an ideal *in vitro* model for evaluation of several RPE functions and for further elucidation of the mechanisms of RPE involvement in the pathogenesis of retinal and choroidal diseases. These cells are potentially useful in cellular transplant therapy to correct hereditary and age-related macular degeneration defects in humans.

Proposed Course

Two of the major problems associated with human RPE cell cultures are (1) progressive loss of pigmentation upon serial passaging of cells and (2) lack of clear intercellular junctions and *in vivo*-like morphological appearance. These changes may be due to cytoskeletal reorganization and partial dedifferentiation. Our immediate goal is to examine the mechanisms by which RPE cultures can be induced to resume *in vivo* characteristics. This will be achieved by selecting specific media composition, the addition of growth and differentiating factors, and/or culturing on suitable extracellular matrix. Development of a fully differentiated RPE cell line is crucial, not only for understanding cellular functions but also for cellular transplant therapy.

Continuing to evaluate the effects of inflammatory cytokines and bacterial endotoxins on RPE cell cultures, we will address three areas: (1) influences of these factors on cellular cytoskeletal organization, intercellular junctions, and adhesion properties; (2) effects on cell functions (e.g., membrane permeability and solute transport and phagocytosis); and (3) characterization of proteins such as growth factors, cytokines, and proteolytic enzymes secreted by RPE in response to various stimuli. These studies are likely to shed light on the role of RPE in the pathophysiology of the retina and choroid, tissues that are in close proximity to and directly influenced by RPE.

NEI Research Program

Retinal Diseases—Inflammatory Diseases, Macular Degeneration, Photoreceptors, and Retinal Pigment Epithelium

Publications

Kutty RK, Nagineni CN, Kutty G, Hooks JJ, Chader GJ, Wiggert B: Transforming growth factor-β increases the expression of heme oxygenase 1 in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1451, 1993.

Nagineni CN, Detrick B, Hooks JJ: Interferon-γ acts synergistically with inflammatory mediators to induce expression of interleukin 6 by human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1020, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00222-08 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunopathology in Eyes With Experimental and Clinical Ocular Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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	Deborah Luyo		Technician	LI, NEI
	Scott M. Whitcup	M.D.	Medical Officer	LI, NEI
	François G. Roberge	M.D.	Visiting Scientist	LI, NEI
	Rachel R. Caspi	Ph.D.	Visiting Scientist	LI, NEI
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	Robert B. Nussenblatt	M.D.	Scientific Director	NEI

COOPERATING UNITS *(if any)*

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.4	3.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The identity and topographic localization of immunocompetent cells, the alteration of surface markers on ocular resident cells, and their cytokines in animals with experimental autoimmune uveoretinitis or endotoxin-induced uveitis (EAU, EIU) and in human ocular tissues with various diseases were analyzed by immunohistochemical studies and *in situ* hybridization. T lymphocytes were the predominant infiltrating cells in EAU, yet both macrophages and polymorphonuclear neutrophils (PMNs) were the predominant infiltrating cells in EIU. Migration of the inflammatory cells from the vessels into the target site is directed by adhesion molecules, which can be expressed on vascular endothelium and other resident cells in the eye. Mast cells appear to participate in the immunopathogenesis of EAU and EIU. T-lymphocyte specificity is directed to small fragments of antigen bound to cell surface major histocompatibility complex (MHC) molecules, which are presented on the surface of specialized antigen-presenting cells. The expression of MHC class II antigens was observed on ocular resident cells such as retinal pigment epithelium (RPE), retinal endothelium, keratocytes, fibroblasts, and ciliary epithelium in rodents. Both the infiltrating cell subpopulation and the expression of class II antigens and adhesion molecules on ocular resident cells can be altered by various immunomodulating agents and cytokines.

Specimens from human ocular tissues with various diseases—such as uveitis, retinal disease, conjunctival and corneal diseases, metabolic genetic diseases, and tumors—are studied using immunohistochemical and *in situ* hybridization techniques as well as light and electron microscopic evaluation. In uveitis, immunocompetent cells and lymphokines are valuable adjuncts to clinical diagnosis, and they are determinants of disease course and prognosis. In nonuveitic conditions, alteration of cellular membrane surface markers and intracytoskeleton of the ocular resident cells may imply damage and abnormalities in these diseases. Elucidating the immunopathological role of the relationships between infiltrating inflammatory or malignant cells and other resident cells in the clinical behavior of various diseases will increase our understanding of human ocular disorders.

Project Description

Objectives

This program is designed to evaluate the clinical manifestation, histopathology, and immunopathology of the ocular tissue when experimental autoimmune uveoretinitis (EAU) and endotoxin-induced uveitis (EIU) are induced and/or modulated by various immunosuppressive agents in various animal species. Ocular tissues obtained from patients with various diseases, including inflammatory and noninflammatory disorders, also are studied. The infiltrating inflammatory cells, ocular resident cells and their products, and various lymphokines and cytokines are examined. The findings will help us understand ocular inflammation and the pathogenesis of each disease examined in humans.

Methods

Clinical examinations include flashlight and slit-lamp examinations as well as examination of the fundus of animals and patients under the dissecting microscope. Pathological examinations include routine histologic techniques for light and electron microscopy, immunofluorescence, avidin-biotin-peroxidase complex methods, and *in situ* hybridization techniques.

Major Findings

We continued to study the immunopathology of various inflammatory cells and ocular resident cells in different experimental models of uveitis. We have observed that, prior to the infiltration of inflammatory cells into the eye, the number of mast cells in the anterior uvea decreases and is consistent with mast cell degranulation in EAU and EIU. This observation suggests that anterior uveal mast cells participate in uveitis by releasing vasoactive amines to alter the integrity of the blood-ocular barrier and amplify the inflammatory process.

Toward understanding the immunopathological process in various experimental uveitides, we have examined the efficacy of different anti-inflammatory mediators and immunomodulating agents in these animal models. For example, we have found that feeding animals rat chow mixed with CGS-13080, a thromboxane synthetase inhibitor, suppresses the development of clinical and histopathological EAU. Inhibition of this enzyme results in a reduction of thromboxane B_2 and an increase of prostaglandin E in the serum, thus altering the inflammatory mediator and suppressing EAU.

Antibodies against adhesion molecules are able to abrogate EAU and EIU because expression of adhesion molecules precedes the flux of inflammatory cells into the eye. The cytokine cascade in the inflammatory process is complicated. Tumor necrosis factor α has a paradoxical role in EIU. Immunosuppressive agents—in particular the inhibitors of T-cell function, such as cyclosporine A, FK 506, and rapamycin, which interfere with the release of lymphokines—are potent and effective medications to treat EAU, a T-cell-mediated autoimmune uveoretinitis.

Using immunopathological techniques, we examine ocular tissues obtained from patients with various ocular diseases to help visualize the pathology and the kinetics of the specific disease process. The findings provide useful information for understanding the pathological mechanisms of the disease, determining the diagnosis, and guiding the subsequent management of patients.

We found collagen dysgenesis in Reis-Buckler corneal dystrophy. The presence of immature collagen type III and poorly developed collagen type I may contribute to the pathogenesis of Reis-Buckler dystrophy. In Cogan-Reese syndrome, we found that the iris nevi are cells that originate in the neural crest and have numerous melanosomes, junctional complexes, and basement membrane. We demonstrated the presence of αB-crystallin, a major lens protein in retinoblastoma, suggesting that αB-crystallin is involved in tumor growth and/or is a marker for general oncogenic "stress" in retinoblastoma. We also have shown the presence of tachyzoites and the role of T lymphocyte in congenital toxoplasmosis.

Using *in situ* hybridization, we detected the RNAs of both interleukins 2 and 4 in the conjunctiva of ocular onchocercal patients, suggesting that Th2 cells and their lymphokines are important for localized host responsiveness to ocular onchocerciasis.

In practice, correct handling and processing of surgical specimens obtained from vitrectomy and/or chorioretinal biopsy can yield important information, in particular, the diagnosis of intraocular large B-cell lymphoma (central nervous system lymphoma) and progressive chorioretinal lesions of unknown etiology. Once the diagnosis is made, the appropriate treatment can be offered.

We are investigating other experimental models, e.g., allergic conjunctivitis, melanin-protein-induced uveitis (EMIU), and acquired toxoplasmosis, and their resemblance to other ocular inflammatory diseases in humans. We hope to better understand the mechanisms of ocular inflammation and evaluate the effects of different therapeutic approaches in these different new models.

Significance to Biomedical Research and the Program of the Institute

Immunopathological findings on experimental uveitides have provided information on various inflammatory cells and ocular resident cells during the process of ocular inflammation. This information helps us to choose and evaluate novel pharmacologic agents and provide better therapeutic intervention of uveitis in humans. Studies of ocular tissues obtained from patients with various disorders have enabled us to gain information on the mechanism, diagnosis, and management of these ocular diseases. This information is useful in treating patients not only with uveitis but also with ocular tumors and congenital disorders.

Proposed Course

Various experimental models, including EAU, EIU, EMIU, allergic conjunctivitis, and acquired toxoplasmosis, will be studied clinically, histopathologically, and immunopathologically in different species and strains. Various pharmacological agents and the role of cytokines, lymphokines, enzymes, and cellular surface markers will be evaluated in these models. Also, we propose continuation of analysis of human specimens in the study of their immunopathogenesis.

NEI Research Program

Retinal and Choroidal Disease—Inflammatory Disorders

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00241-07 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunopathology of Ocular Diseases in Humans

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Chi-Chao Chan	M.D.	Chief, Section on Immunopathology	LI, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Qian Li	M.D.	Visiting Fellow	LI, NEI
	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
	Raymond DeBarge	M.D.	Senior Staff Fellow	LI, NEI
	Scott M. Whitcup	M.D.	Staff Medical Officer	LI, NEI
	Juan Lopez	M.D.	Visiting Associate	LI, NEI
	Miguel Burnier	M.D.	Visiting Scientist	LI, NEI
	Richard Fenton	M.D.	Staff Fellow	LI, NEI
	Dev Shah	M.D.	Visiting Associate	LI, NEI

COOPERATING UNITS *(if any)*

Department of Ophthalmology, Armed Forces Institute of Pathology (Ian W. McLean, M.D.); University of Minnesota, Department of Ophthalmology (Edward J. Holland, M.D.); L'Hôpital de la Pitié, Paris, France (Phuc LeHoang, M.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated and combined with Project No. Z01 EY 00222-08 LI.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00264-04 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cytokines and Ocular Antigens in the Eye

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	LI, NEI
	Igal Gery	Ph.D.	Head, Section on Experimental Immunology	LI, NEI
	Qian Li	M.D.	Visiting Fellow	LI, NEI
	Louis Kasner	M.D.	Fellow	LI, NEI

COOPERATING UNITS *(if any)*

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Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated and combined with project number Z01 EY 00222-08 LI.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00268-03 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Diagnosis and Treatment of Human Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Scott M. Whitcup	M.D.	Staff Medical Officer	LI, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
	Chi-Chao Chan	M.D.	Medical Officer	LI, NEI

COOPERATING UNITS *(if any)*

Department of Medicine, The Johns Hopkins University, Baltimore, MD (David R. Moller, M.D.)

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Laboratory of Immunology

SECTION

Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to develop improved methods for diagnosing and treating human uveitis. Three studies of diagnosis are as follows: (1) We examine biopsy and pathology specimens from patients with uveitis and AIDS to develop improved diagnostic tests and to understand better the pathophysiology of inflammatory eye disease. Ongoing studies of intraocular lymphoma show that multiple vitrectomies or lumbar punctures are required to diagnose about one-third of the patients. Appropriate, prompt handling of pathology specimens by an experienced cytopathologist remains critical to making the correct diagnosis. (2) To improve methods for diagnosing ocular sarcoidosis, we test lacrimal gland and conjunctival biopsies for the presence of interferon-gamma; Kveim antigen; interleukins 2, 3, 4, 6, and 8; and T-cell receptors believed to be specific for this disease. This year we retrospectively reviewed 46 patients with biopsy-proven sarcoidosis and 21 with uveitis. In patients with ocular involvement, the most sensitive diagnostic test was the pulmonary diffusing capacity (DLCO), which diminished in 78% of patients tested. Corticotropin-releasing hormone tests are performed on patients with uveitis to determine whether a defective hypothalamic-pituitary-adrenal axis is associated with increased risk for autoimmune ocular inflammatory disease. (3) Our study of animals with endotoxin-induced uveitis (EIU) showed that tumor necrosis factor alpha causes a paradoxical exacerbation of ocular disease.

In the area of treatment, we have three projects: (1) We are continuing a masked, randomized crossover study to compare acetazolamide with placebo for the treatment of uveitic cystoid macular edema; to date 31 patients have been recruited. (2) Topically applied FK 506 was used to treat EIU in the rat. Ocular inflammation was reduced significantly in animals treated with topical FK 506 (0.3% and 0.05%) when compared with control animals, a finding that may be useful in the treatment of acute ocular inflammation in humans. (3) The optimal therapy for intraocular lymphoma remains unclear; however, previous studies suggest that untreated patients die within 1 year of diagnosis. Retrospective review of 11 patients with intraocular lymphoma treated with radiation, chemotherapy, or both showed substantial treatment-related mortality. In a joint protocol with the National Cancer Institute, we now are investigating alternative treatment regimens for central nervous system lymphoma.

Project Description

Additional Personnel

Emily Chew	M.D.	Visiting Scientist, BEP, NEI
Frederick Ferris, III	M.D.	Chief, Clinical Trials Branch, BEP, NEI
George P. Chrousos	M.D.	Diabetes Epidemiology Branch (DEB), National Institute of Child Health and Disease (NICHD)
Daniel Martin	M.D.	Senior Staff Fellow, LI, NEI
George Mastorakos	M.D.	Visiting Scientist, DEB, NICHD
Igal Gery	Ph.D.	Deputy Chief, LI, NEI

Clinical Protocol Numbers

90-EI-132
91-EI-30
91-EI-139
92-EI-0070

Objectives

The goal of this study is to develop better methods for the diagnosis and treatment of human uveitis. We also are interested in defining the pathophysiology of inflammatory eye diseases by analyzing human tissue and animal models of uveitis.

Methods

Diagnosis of Uveitis

1. To improve the diagnostic yield of conjunctival and lacrimal gland biopsies for sarcoidosis, we are examining tissue specimens using immunohistochemical staining. Conjunctival and lacrimal gland biopsies will be performed on 10 patients with known sarcoidosis and snap frozen in O.C.T.® Immunohistochemical staining will be performed using an avidin-biotin-peroxidase complex. Primary monoclonal antibodies against T-cell markers, T-cell receptors, Kveim antigen, and various interleukins will be applied. The results will be compared with those of biopsies from patients with other uveitic conditions, such as Behçet's disease, to determine the specificity of these results. We also have retrospectively reviewed the records of patients with biopsy-proven sarcoidosis to determine the sensitivity of current tests obtained to diagnose sarcoidosis.

2. Intraocular lymphoma often masquerades as an idiopathic uveitis, which delays the start of appropriate therapy. We continue to collect data on patients diagnosed with intraocular lymphoma.

3. We are performing corticotropin-releasing hormone tests to access the hypothalamic-pituitary-adrenal axis in patients with autoimmune uveitis. Subnormal cortisol production in response to this hormone may predispose patients to the development of autoimmune disease.

4. The pathophysiology of endotoxin-induced uveitis (EIU) is being studied using immunohistochemistry, histology, and monoclonal antibodies against various cytokines.

Treatment of Uveitis

1. The efficacy of acetazolamide for the treatment of uveitis-associated macular edema is being evaluated in a masked, crossover study comparing acetazolamide with placebo. Visual acuity and the height of the macular edema measured by fluorescein angiography are the primary endpoints.

2. We are testing the efficacy of topically applied FK 506, a new immunosuppressive agent, for the treatment of acute anterior uveitis, using the animal model of EIU in the rat. Histologic evidence of intraocular inflammation and aqueous humor protein concentrations are compared between treated and control animals.

3. In an investigation of treatment for patients with intraocular lymphoma, we are reviewing both morbidity and mortality. In addition, we are participating in a joint protocol with the National Cancer Institute to study chemotherapy on non-Hodgkin's lymphoma arising in the central nervous system (CNS) or the eye.

4. We are comparing trabeculectomy combined with subconjunctival 5-fluorouracil to the Molteno implant for the treatment of glaucoma secondary to uveitis.

Major Findings

1. We retrospectively reviewed 46 patients with biopsy-proven sarcoidosis, 21 with uveitis. In patients with ocular involvement, only 61% had abnormal chest x-rays; 36% had an elevated angiotensin-converting enzyme. The most sensitive

diagnostic test was the pulmonary diffusing capacity, which was diminished in 78% of the patients tested. There was no statistically significant difference between the test results of sarcoidosis patients with and without uveitis. Among 21 uveitis patients, 14 (67%) had visual acuity of 20/40 or worse in at least one eye. Poor visual acuity (20/200 or worse) was predominantly caused by secondary glaucoma.

2. Examination of the use of topical FK 506 for the treatment of EIU showed that the mean anterior chamber cell count per microliter and the median histologic grade of ocular inflammation (scale of 0 to 4) were significantly decreased in rats treated with topical FK 506 0.05% and FK 506 0.3% when compared with those of placebo-treated rats. The blood levels of FK 506 in rats treated with topical 0.05% and 0.3% FK 506 were 1.2 and 2.9 mg/ml, respectively—more than tenfold lower than levels obtained with systemic therapy at a dose of 1 mg/kg.

3. Retrospective review of 11 patients with intraocular lymphoma treated with radiation, chemotherapy, or both showed that 5 patients died a mean of 21 months after diagnosis while 6 have survived a mean of 33 months. We performed autopsies on three of the five patients who died. Interestingly, no residual lymphoma was found in any of the three, whose deaths were felt to result from treatment-related complications, predominantly severe leukoencephalopathy. This substantial treatment-related mortality suggests that improved therapeutic regimens are needed. We are currently involved in a joint protocol with the National Cancer Institute, investigating alternative treatment regimens for CNS lymphoma.

Significance to Biomedical Research and the Program of the Institute

Uveitis accounts for about 10% of visual impairment in the United States. A major goal of the NEI is to improve the methods for diagnosing and treating uveitis in an attempt to preserve useful vision in patients with inflammatory eye disease.

Proposed Course

We will continue patient recruitment for the clinical trials of cystoid macular edema, corticotropin-releasing hormone, sarcoidosis, uveitic glaucoma, and intraocular lymphoma. We have completed our initial studies, showing the effectiveness of topically applied FK 506 for the treatment of EIU, and we are planning to investigate the use of liposome-bound FK 506 to improve ocular penetration of topically applied compounds. In addition, studies on the effect of cytokines on ocular inflammatory disease will continue.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Chan C-C, Li Q, Brezin AP, Whitcup SM, Egwuagu C, Otteson EA, Nussenblatt RB: Immunopathology of ocular onchocerciasis. 3. Th-2 helper T cells in the conjunctiva. *Ocular Immunol Inflam* 1:71-77, 1993.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00269-03 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ocular Toxicity of 2′,3′-Dideoxyinosine (ddI)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Scott M. Whitcup	M.D.	Staff Medical Officer	LI, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI

COOPERATING UNITS *(if any)*

Pediatric Branch, National Cancer Institute (Philip A. Pizzo, M.D.); Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases (Clifford H. Lane, M.D.); Clinical Oncology Program, National Cancer Institute (Robert Yarchoan, M.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

2′,3′-Dideoxyinosine (ddI), a purine analog with antiretroviral activity currently used to treat patients with AIDS (acquired immune deficiency syndrome), is being used to treat both adults and children in clinical protocols at the National Institutes of Health. The purpose of this study is to follow prospectively patients treated with ddI for the development of ocular complications secondary to drug toxicity. Ninety-five children with symptomatic (CDC class P-2) HIV (human immunodeficiency virus) infection were enrolled in a phase I/II study to assess the safety and antiretroviral activity of ddI. Five children developed peripheral atrophy of the retinal pigment epithelium during ddI therapy. The two children with the most severe retinal atrophy were enrolled in the study at the highest dose level studied (540 mg/m^2/day). Electro-oculograms were abnormal in one of three patients with retinal toxicity who could be tested. A group of 75 adults treated with ddI are being followed with periodic fundus examinations and electro-oculograms. During the past year similar retinal lesions were found in one adult patient treated with ddI.

Project Description

Additional Personnel

Daniel Martin	M.D. Senior Staff Fellow, LI, NEI
Margaret Cheung	M.D. Senior Staff Fellow
David Parks	M.D. Senior Staff Fellow
John J. Hooks	Ph.D. Head, Section on Immunology and Virology, LI, NEI
Caroline Percopo	M.S. Biologist, LI, NEI

Objectives

The goal of this study is to monitor patients treated with 2′,3′-dideoxyinosine (ddI) for the development of ocular complications.

Methods

Every 3-4 months patients treated with ddI are given complete eye examinations, including dilated ophthalmoscopy and fundus photography of any abnormal retinal findings. Patients treated with the higher dosages of ddI also receive periodic electro-oculograms to assess the electrophysiologic function of the retinal pigment epithelium (RPE).

Major Findings

1. Five children have now developed peripheral atrophy of the RPE during ddI therapy. The lesions are scalloped areas of RPE atrophy with hyperpigmented borders. They occur predominantly in the midperiphery of the fundus in both eyes. These retinal lesions slowly progress if ddI therapy is continued, but central visual acuity has remained unaffected. During the past year no discrete retinal lesions have developed in any other children treated with ddI.

2. One adult patient treated with ddI developed progressive, well-circumscribed atrophic retinal lesions of the peripheral RPE, similar to those in the children. The lesions appeared after 32 months of ddI treatment; the total dosage received was 264 g (approximately 3.3 g/kg). Adjacent areas of RPE mottling also were seen. Visual acuity and electro-oculography were normal in this patient.

Significance to Biomedical Research and the Program of the Institute

ddI is a drug with *in vitro* and *in vivo* activity against HIV (human immunodeficiency virus) infection. One mission of the NEI is to monitor patients for the development of ocular toxicity and to assess the effect such toxicity has on vision.

Proposed Course

We will continue to follow all patients treated with ddI at the NIH for signs of ocular manifestations of ddI toxicity or HIV infection. We are performing serial electro-oculograms in adults treated with ddI.

NEI Research Program

Retinal Diseases—Photoreceptors and Retinal Pigment Epithelium

Publications

Nguyen B-Y, Shay LE, Wyvill KM, Pluda JM, Brawley O, Cohen RB, Whitcup SM, Venzon DJ, Broder S, Yarchoan R: A pilot study of sequential therapy with zidovudine (AZT) plus acyclovir,dideoxyinosine, dideoxcytidine in patients with severe human immunodeficiency virus infection. *J Infect Dis* 168:810-817, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00270-03 LI

PERIOD COVERED

October I, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell Adhesion Molecules in Ocular Inflammation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Scott M. Whitcup	M.D.	Staff Medical Officer	LI, NEI
Others:	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI

COOPERATING UNITS *(if any)*

Biochemical and Molecular Pathology, Merck Sharp & Dohme Research Laboratories (Hugh Rosen, M.D.); Immunology Section, Roberts Pharmaceutical Corporation (Ron Harning, Ph.D.); Department of Ophthalmology, Kurume University School of Medicine, Fukuoka, Japan (Manabu Mochizuki, M.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunopathology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cell adhesion molecules are surface proteins important for antigen sensitization and the migration of leukocytes to sites of inflammation. We are studying the expression of cell adhesion molecules in ocular inflammation, investigating the blocking of cell adhesion molecules as a treatment for uveitis and other ocular inflammatory diseases, and examining the effect of immunosuppressive agents on cell adhesion molecule expression in eyes with experimental autoimmune uveitis (EAU).

We previously showed that intercellular adhesion molecule 1 (ICAM-1) is expressed in eyes with EAU before the infiltration of inflammatory cells. Further experiments showed that monoclonal antibodies against ICAM-1 and its counter-receptor lymphocyte function-associated antigen 1 (LFA-1) inhibit EAU development in mice.

We demonstrated that cell adhesion molecules are important for both antigen sensitization and inflammatory cell infiltration into the eye, with additional sets of experiments showing the following: (1) that monoclonal antibodies against both ICAM-I and LFA-1 will prevent inflammatory cell infiltration of the eye induced by endotoxin, and importantly that these antibodies can inhibit ocular inflammation even when administered after signs of inflammation have been noted; and (2) that monoclonal antibodies against ICAM-1 and LFA-1 can inhibit *in vitro* proliferation of a uveitogenic cell line by interfering with the interaction between lymphocytes and antigen-presenting cells. These results suggest that cell adhesion molecules play an important role in the development of uveitis and that blockage of cell adhesion molecules may provide a new therapeutic approach for patients with inflammatory eye disease.

Finally, we examined the effect of immunosuppressive agents on the expression of cell adhesion molecules in animals with EAU. Ocular expression of cell adhesion molecules was delayed and downregulated in animals treated with corticosteroids and cyclosporine following immunization with retinal S-antigen. Downregulation of cell adhesion molecule expression may be one of the mechanisms by which immunosuppressive agents inhibit ocular inflammation.

Project Description

Additional Personnel

Rachel Caspi	Ph.D.	Visiting Associate, LI, NEI
Igal Gery	Ph.D.	Deputy Chief, LI, NEI
Qian Li	M.D.	Visiting Fellow, LI, NEI

Objectives

The goal of this project is to examine the role of cell adhesion molecules in ocular inflammation. We are studying the expression of cell adhesion molecules in eyes with uveitis and examining the effect of blocking these adhesion molecules on the development of ocular inflammatory disease. We also are investigating the role of cell adhesion molecules in antigen sensitization. By blocking cell adhesion molecules or preventing the expression of these surface proteins, we hope to be better able to treat patients with ocular inflammatory disease.

Methods

Animal models of ocular inflammation.—Endotoxin-induced uveitis (EIU) is induced by injecting 100 μg of *Salmonella typhimurium* endotoxin into one footpad of a Lewis rat or 200 μg into one footpad of a C3H-hen mouse. Experimental autoimmune uveitis (EAU) in mice is induced by immunizing B10.A mice with 50 μg of interphotoreceptor retinoid-binding protein (IRBP) in complete Freund's adjuvant, with pertussis toxin injected intraperitoneally.

Histology and immunohistochemistry of ocular inflammation.—Enucleated animal eyes and human ocular tissue are immediately snap frozen and embedded in O.C.T.® The expression of cell adhesion molecules and the presence of cytokines are then assessed by immunohistochemical staining with avidin-biotin-peroxidase complex (ABC) on frozen sections of ocular tissue. Eyes also are embedded in methyl methacrylate, and 4-μm sections are examined for histologic evidence of inflammation.

Treatment of ocular inflammation by blocking cell adhesion molecules.—In an attempt to inhibit the development of ocular inflammation, we treated animals with intraperitoneal injections of monoclonal antibodies against ICAM-1 or LFA-1 before the induction of either EIU and EAU.

Effect of monoclonal antibodies against ICAM-1 or LFA-1 on cell proliferation of a uveitogenic cell line.—Mouse anti-rat ICAM-1 (CD54) monoclonal antibody (mAb), designated IA29, and mouse anti-rat LFA-1 (CD11a) mAb, designated WT.1, were kindly provided by Dr. Miyasaka (Tokyo, Japan). mAbs were incubated with irradiated Lewis rat thymocytes (antigen-presenting cells [APCs]) at concentrations of 10, 1, 0.1, and 0 μg/ml for 2 hours. These cells were then added to lymphocyte cultures comprised of CD4+ T cells of a highly uveitogenic cell line, sensitized against IRBP-derived peptide R15 (sequence 1181-1191), and stimulated with peptide R15 (1 or 0.01 μM) or with concanavalin A (con A). The cultures, which consisted of 2×10^4 lymphocytes and 1×10^5 or 5×10^5 APCs in 0.2 ml of medium, were processed as previously detailed (*Cell Immunol* 122:251, 1989).

Effect of corticosteroids and cyclosporine A (CsA) on the expression of cell adhesion molecules in eyes with EAU.—EAU was induced in 36 female Lewis rats by injecting into one hind footpad 30 μg of retinal S-antigen in complete Freund's adjuvant containing 0.25 mg of *Mycobacterium tuberculosis.* Rats were then treated with daily intramuscular injections of 0.2 mg/kg methylprednisolone (MP), 3 mg/kg CsA, or olive oil as a control. Rats were sacrificed 0, 7, 10, 14, 21, and 28 days after immunization. Each right eye was processed for routine histology, and each left eye was immediately snap-frozen for immunohistochemical staining, using an avidin-biotin-peroxidase technique and primary antibodies against ICAM-1 (CD54), LFA-1 alpha (CD11a), E-selectin, major histocompatibility complex (MHC) class II antigens (RT1B and RT1D), CD4+ T cells (W3/25), and CD8+ T cells (OX8). Slides were then graded by two masked observers.

Major Findings

1. When treatment was given at the time of endotoxin injection, the mean number of inflammatory cells infiltrating the eye on histologic sections was 469.2 ± 51.9 (standard error of the mean [SEM]) for controls, 13.8 ± 2.6 for rats receiving anti-ICAM-1 mAb ($p < 0.001$), and 195.8 ± 48.8 for rats receiving anti-LFA-1 mAb ($p < 0.001$). When treated after the start of inflammatory disease, the mean number of infiltrating inflammatory cells ± SEM was 273.0 ± 30.7 for controls, 6.4 ± 1.7 for rats receiving anti-

ICAM-1 mAb ($p < 0.001$), and 54.2 ± 7.6 for rats receiving anti-LFA-1 mAb ($p < 0.001$). The mean numbers of cells per microliter of aqueous humor were 1867.6 ± 321.8 for controls, 21.7 ± 5.3 for rats receiving anti-ICAM-1 mAb ($p < 0.001$), and 295.1 ± 71.2 for rats receiving anti-LFA-1 mAb ($p < 0.001$). Treatment with mAbs against ICAM-1 and LFA-1 significantly inhibited the development of EIU and was effective in treating clinically evident ocular inflammatory disease.

2. In mice with EAU, ocular inflammation, graded clinically by examination of the fundus 14 and 21 days after immunization, was significantly decreased in animals treated with anti-ICAM-1 ($p < 0.01$ at days 14 and 21) and with anti-LFA-1 antibody ($p < 0.01$ at days 14 and 21).

3. In cultures containing 5×10^5 APCs, anti-LFA-1 mAb (10 µg/ml) inhibited the lymphocyte response to 1 and 0.01 µM of R15 by 58% and 74%, respectively. mAbs against ICAM-1 were less inhibitory, reducing the responses to R15 by 23% and 30% for doses of anti-LFA and R15 mAb, respectively. Decreasing the APC concentration had little effect on the antibody activity, while decreasing the mAb concentration to ≤1 µg/ml almost completely eliminated their inhibitory capacity. mAbs against LFA-1 and ICAM-1 inhibited the interaction between APCs and lymphocytes of a uveitogenic cell line. We propose that this activity plays a major role in the inhibition of EAU in animals treated with mAbs against LFA-1 and ICAM-1.

4. By 14 days after immunization, ICAM-1, E-selectin, and MHC class II antigens were strongly expressed on the vascular endothelium of the iris, ciliary body, choroid, and retina of control rats; infiltrating lymphocytes expressing LFA-1 also were noted in these eyes. In contrast, 28 days after immunization, rats treated with MP and CsA still had only mild expression of ICAM-1, E-selectin, and MHC class II antigens, and few infiltrating lymphocytes were noted on histologic sections. Ocular expression of cell adhesion molecules was delayed and down-regulated in animals treated with MP and CsA following immunization with S-antigen. Expression of class II antigens and infiltration with inflammatory cells also were diminished in eyes with decreased expression of cell adhesion molecules. Down-regulation of cell adhesion molecule expression may be one of the mechanisms by which corticosteroids and CsA inhibit ocular inflammation.

Significance to Biomedical Research and the Program of the Institute

One major mission of the NEI is to understand the mechanisms of sight-threatening eye diseases so that new and effective therapies can be developed. The expression of cell adhesion molecules appears to be a fundamental mechanism in the development of intraocular inflammation. With this understanding, we hope to develop new anti-inflammatory therapy for ocular inflammation, which accounts for approximately 10% of the visual impairment in the United States.

Proposed Course

We plan to continue our experiments on the expression of cell adhesion molecules in eyes with ocular inflammatory diseases, including uveitis, corneal disease, and uveitic glaucoma. We are examining the roles of additional cell adhesion molecules such as VCAM-1 and VLA-4 in ocular inflammation. In addition, we plan to study the pharmacokinetics of antibodies against cell adhesion molecules, administered topically or intraocularly, to determine the feasibility of local therapy.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Lai JC, Chan C-C, LI Q, Whitcup SM: Treatment with corticosteroids and cyclosporine A inhibits the expression of cell adhesion molecules in experimental autoimmune uveitis (EAU). *Invest Ophthalmol Vis Sci* 34(4)(suppl):1206, 1993.

Vistica B, Gery I, Chan C-C, Nussenblatt RB, Whitcup SM: Anti-ICAM-1 and anti-LFA-1 monoclonal antibodies (mAbs) inhibit in vitro proliferation of a uveitogenic cell line. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1144, 1993.

Whitcup SM, DeBarge LR, Caspi RR, Harning R, Nussenblatt RB, Chan C-C: Monoclonal antibodies against ICAM-1 (CD54) and LFA-1 (CD11a/CD18) inhibit experimental autoimmune uveitis. *Clin Immunol Immunopathol* 67:143-150, 1993.

Whitcup SM, DeBarge LR, Rosen H, Nussenblatt RB, Chan C-C: Monoclonal antibody against CD11b/CD18 inhibits endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci* 34:673-681, 1993.

Whitcup SM, Hikita N, Shirao M, Mochizuki M, Nussenblatt RB, Chan C-C: Effect of monoclonal antibodies against ICAM-1 (CD54) and LFA-1 alpha (CD11a) in the prevention and treatment of endotoxin-induced uveitis (EIU). *Invest Ophthalmol Vis Sci* 34(4)(suppl):1143, 1993.

Whitcup SM, Nussenblatt RB, Price FW Jr, Chan C-C: Expression of cell adhesion molecules in corneal graft failure. *Cornea*, 12:475-480, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00184-11 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular and Immunogenetic Mechanisms in Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Rachel R. Caspi	Ph.D.	Visiting Scientist Acting Head, Section on Immunoregulation	LI, NEI
Others:	Phyllis Silver	B.S.	Biologist	LI, NEI
	Luiz Rizzo	M.D.	Visiting Associate	LI, NEI
	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI

COOPERATING UNITS *(if any)*

Laboratory of Immunobiology, Rega Instituut, Katholieke Universiteit, Leuwen, Belgium (A. Billiau, M.D.; H. Heremans, Ph.D.); Arthritis and Rheumatism Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases (Ronald L. Wilder, M.D., Ph.D.); Bone Marrow Transplantation Unit, National Cancer Institute (Frances Hakim, Ph.D.); Research and Development, Wills Eye Hospital, Philadelphia, PA (Larry A. Donoso, M.D., Ph.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.6	2.6	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cellular mechanisms in ocular immunologically mediated disease are being studied in animal models of experimental autoimmune uveoretinitis (EAU). Rats and mice are immunized with retinal-derived antigens, or synthetic peptides representing fragments of these antigens, to induce EAU. Susceptibility to disease is being evaluated in various strains of known genetic makeup in the hope of delineating the hereditary mechanisms that predispose to uveitis. EAU in rats and mice serves as a template for the evaluation of new drugs and compounds as well as for the study and characterization of the participating cells and their factors. *In vivo* functional long-term T-cell lines and clones are developed from lymphoid organs of rats and mice immunized with uveitogenic ocular proteins. The functional properties and antigen receptors of these cells are studied to develop strategies for *in vivo* targeting of the autoimmune cells. The goal of these studies is to identify the immunogenetic factors predisposing to uveitic disease, learn about the pathogenic mechanisms involved, characterize the immunoreactive cells and their mediators, and finally to utilize this knowledge for designing rational approaches to immunotherapy.

Project Description

Additional Personnel

Robert B. Nussenblatt	M.D.	Chief, LI, NEI
Charles E. Egwuagu	Ph.D.	Staff Fellow, LI, NEI
Igal Gery	Ph.D.	Head, Section on Experimental Immunology, LI, NEI

Objectives

The development and study of animal models of experimental ocular autoimmune disease permits the study of cellular and genetic factors that may be involved in ocular autoimmunity in a general sense. Experimental autoimmune uveitis (EAU) in rats and mice serves as a template for the evaluation of new drugs and compounds as well as for the study and characterization of the participating cells and their factors. Long-term maintenance of T cells *in vitro* permits the investigators to separate and selectively grow various T-cell subsets. The goals are (1) to continue to establish and characterize the murine EAU model because the mouse offers some important advantages over other rodents as a model of EAU; (2) to use the EAU model in rodents for the study of cellular mechanisms in ocular autoimmunity; this is done in large part by establishing and using retinal antigen-specific T-cell lines and clones, permitting us to identify and characterize cells capable of ocular immunomodulation, learn about migration and localization of autoimmune lymphocytes, and study their interactions with other lymphoid and nonlymphoid cells in eliciting effector mechanisms; (3) to use the EAU model as a template for the development of immunotherapeutic approaches designed to target autoimmune lymphocytes directly or to disrupt specific stages in the autoimmune inflammatory cascade; and (4) to use the murine EAU model for the study of various genetic mechanisms controlling susceptibility to ocular autoimmune disease. The study and understanding of these parameters will help not only in the development of new therapies but possibly in the prevention of ocular disease.

Methods

Rats and mice of various strains are immunized with purified S-antigen (S-Ag) or interphotoreceptor retinoid-binding protein (IRBP) in complete Freund's adjuvant or with various pathogenic peptides derived from these proteins. After disease development, eyes are processed for histopathology and examined for disease, and lymphoid cells are taken from the blood, lymph nodes, or eyes. Cells thus obtained are placed in culture either with mitogen or with the retinal antigen with which the donor animal was immunized. Responses of the immune cells are studied.

Cells also are expanded in culture and used in attempts to transfer EAU to nonimmune animals in order to find out the cell population responsible for disease induction. Long-term cell lines are developed and in some cases are cloned by either the soft agar bilayer or the limiting dilution technique. These lines or clones are then tested for functional characteristics such as the ability to induce ocular disease, production of soluble mediators, expression of various cell surface molecules, response to therapeutic agents, and interactions with other cells in culture.

Major Findings

We previously had studied the importance of "nonspecific" T-cell recruitment in the immunopathogenesis of uveitis by using congenitally athymic Lewis rats (LEW.rnu/rnu), which are deficient in functional endogenous T cells but are otherwise syngeneic with the euthymic Lewis rats that develop characteristically severe EAU. The uveitogenic stimulus was delivered in the form of phenotypically and functionally homogeneous pathogenic T-cell lines specific to the major pathogenic epitope of either the intracellular photoreceptor protein, S-Ag, or the extracellular photoreceptor matrix protein, IRBP. Previous data indicated that, depending on the T-cell line used, EAU in athymic rats was either drastically reduced in severity or absent. Susceptibility was restored when the athymic animals were reconstituted with immunocompetent T cells from syngeneic euthymic donors.

We now have shown that the severity of inflammation and tissue damage are correlated with the proportion of lymphocytes in the intraocular infiltrate: The infiltrate in euthymic rats was predominantly lymphocytic, with smaller numbers of monocyte-macrophages and even fewer neutrophils, whereas the sparse infiltrate in athymics was largely monocytic and had a relatively high proportion of neutrophils and eosinophils. Reconstituted animals had an intermediate histological picture with respect

to the infiltrating cell types and disease severity. Our results indicate that recruited nonspecific T cells play a major role in the pathogenesis of disease.

Furthermore, the data suggest that the extent of dependence on recruitment may be influenced by the antigenic specificity of the T-cell line and could be connected to the "accessibility" of the target antigen *in vivo*. This is the first direct demonstration that recruitable "nonspecific" T lymphocytes are necessary for the expression of the disease itself.

In collaboration with Dr. Charles Egwuagu, we are continuing to study the T-cell receptor (TCR) genes of these lines and clones on the molecular level. The data indicate that TCR variable-region gene usage in uveitis differs from that reported for some other autoimmune diseases and may be more heterogeneous. We currently are studying TCR expression in the inflamed eyes of Lewis rats immunized with various retinal antigens or adoptively transferred with T-cell lines of the appropriate specificity.

Because our previous findings with athymic rats suggest that the majority of lymphocytes infiltrating the eye are likely to be recruited cells, we are looking at the earliest stages of disease induction as well as at cells that infiltrate the eye in athymic nude rats injected with pathogenic T cells. The results appear to confirm our previous findings: TCR Vβ8.2 may be a pathogenic clonotype in S-Ag-EAU, whereas TCR Vβ8.3 may be one of the pathogenic clonotypes in IRBP-EAU. The results also indicate that additional clonotypes such as Vβ14 may be involved. These findings could impact on the development of therapeutic strategies designed to specifically target the pathogenic cells through their T-cell receptors.

In the mouse model of EAU, we have developed a pathogenic T-cell line specific to the whole IRBP molecule in the B10.A strain of mice ($I\text{-}A^k$). The line was developed from draining lymph nodes of IRBP-immunized mice using a protocol similar to that used for the derivation of uveitogenic T-cell lines in the rat (alternating cycles of stimulation with antigen and expansion in interleukin 2). After the fourth weekly stimulation with IRBP, we tested the line for pathogenicity and found that it induced uveitis at 5×10^6 cells per mouse. The line elaborated an unrestricted lymphokine profile, suggesting that both Th1-type and Th2-type cells were present.

With continued stimulations in culture, the cell line became progressively more pathogenic. After 16 cycles the cell line was pathogenic at cell numbers as low as 10^5 cells per mouse. The TCR profile of the line also changed with time in culture, with progressive enrichment in Vβ8.2 and Vβ6 TCR-expressing cells (64% and 16%, respectively), suggesting that Vβ8.2 and Vβ6 may represent pathogenic clonotypes in IRBP-EAU in the B10.A mouse. The line currently is being cloned to test this hypothesis and to further characterize the pathogenic cells with respect to their Th1-like or Th2-like identity. Another IRBP-specific pathogenic T-cell line was developed, using a similar culture protocol, from eyes of uveitic B10.A mice. The line was pathogenic at 5×10^6 cells per mouse. These results suggest that the pathogenic cells infiltrate and are physically present in the eye during uveitis.

In all animal species, as well as in humans, the genetic makeup of an individual determines the regions of the uveitogenic protein molecule that evoke an autoimmune response. It is important to study the nature of these epitopes and their relation to different major histocompatibility complex types because the findings could potentially be extrapolated to the situation in humans. In collaboration with Dr. Larry Donoso (Wills Eye Hospital, Philadelphia), who synthesized overlapping peptides representing the entire sequence of the human IRBP molecule, we are engaged in an ongoing effort to identify epitopes that are pathogenic in the three previously identified susceptible mouse H-2 haplotypes, namely, $H\text{-}2^k$, $H\text{-}2^r$, and $H\text{-}2^b$. The peptides are being systematically screened by immunizing animals from strains representing the three susceptible haplotypes. Peptides that cause EAU are being studied further with respect to the identity of the minimal pathogenic sequence, immunodominance, and the fine specificity of the response. Pathogenic T-cell lines are raised to these peptides, and their TCR usage is studied.

Peptide LRHNPGGPSSAVPLLLSYFQ, representing a highly conserved sequence in the IRBP molecule and spanning amino acids 461-480, was found to be pathogenic in C57BL/10, but not in the other mouse strains. The disease scores obtained with the peptide (50-250 μg) were lower than those obtained with the whole IRBP molecule (50-100 μg). A lymphocyte line specific to the peptide was able to

adoptively transfer low-grade uveitis to syngeneic recipients. Mice immunized with the peptide, or with whole IRBP, had positive DTH to the immunizing, but not to the reciprocal, antigen. Lymphocytes of IRBP-immunized mice did not proliferate *in vitro* in response to the peptide; however, positive lymphocyte responses to IRBP could sometimes be detected in peptide-immunized mice and the peptide-specific lymphocyte line proliferated *in vitro* to IRBP. Thus, peptide 461-480 appears to contain an epitope pathogenic to mice of the H-2^b, but not H-2^k or H-2^r, haplotypes. The low pathogenicity of peptide 461-480 in comparison to that of whole IRBP, as well as its lack of immunological recognition by IRBP-immunized mice, *in vivo* or *in vitro*, suggests that it may be a minor, immunologically nondominant epitope. This is the first uveitogenic epitope described for the mouse EAU model. Several additional sites, pathogenic for the other haplotypes, have been tentatively identified and are being studied.

Significance to Biomedical Research and the Program of the Institute

It has become increasingly clear that the cellular mechanisms and possibly the genetic mechanisms observed in animal models of uveitis reflect the mechanisms that operate in ocular immune-mediated disease in humans. The identification and characterization of the cells involved in ocular autoimmunity, and of their functions, will provide new understanding of inflammatory ocular diseases. Successful immunomodulation of EAU in animal models usually has served as a good predictor of the clinical success of a given therapeutic modality. Continued study of basic mechanisms involved in the immunopathogenesis of uveitis in animal models will aid in the development of novel immunotherapeutic approaches for the control of uveitis in humans.

Proposed Course

This project will continue so that more information about the basic mechanisms in experimental uveitis may be obtained.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

Caspi RR. Experimental autoimmune uveoretinitis in rats and mice, in Cohen I, Miller A (eds): *Guidebook to Animal Models for Autoimmune Diseases.* Academic Press, in press.

Caspi RR: Immunogenetic aspects of clinical and experimental uveitis. *Reg Immunol* 4:321-330, 1992.

Caspi RR, Chan C-C, Fujino Y, Najafian F, Grover S, Hansen CT, Wilder RL: Recruitment of naive T cells plays a pivotal role in the pathogenesis of experimental autoimmune uveoretinitis. *Invest Ophthalmol Vis Sci* 34(4)(suppl):902, 1993.

Caspi RR, Chan C-C, Fujino Y, Najafian F, Grover S, Hansen CB, Wilder RL: Recruitment of antigen-nonspecific cells plays a pivotal role in the pathogenesis of a T-cell-mediated organ-specific autoimmune disease, experimental autoimmune uveoretinitis. *J Neuroimmunol* 47:177-188, 1993.

Caspi R, Nussenblatt R: Natural and therapeutic control of ocular autoimmunity—rodent and man, in Kazatchkine M, Roitt I (eds): *Autoimmunity.* Wiley and Sons, Inc, in press.

Egwuagu CE, Mahdi RM, Gery I, Nussenblatt RB, Caspi RR: Evidence for selective accumulation of Vβ8+ T lymphocytes in experimental autoimmune uveoretinitis induced by two different retinal antigens. *J Immunol* 151:1627-1636, 1993.

Mahdi RM, Caspi RR, Nussenblatt RB, Gery I, Egwuagu CE: Selective accumulation of Vβ8+ T lymphocytes in EAU. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1144, 1993.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00218-08 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ocular Manifestations of the Acquired Immune Deficiency Syndrome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
Others:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Scott Whitcup	M.D.	Senior Staff Fellow	LI, NEI
	Margaret Cheung	M.D.	Senior Staff Fellow	LI, NEI
	David Parks	M.D.	Senior Staff Fellow	LI, NEI
	Dan Martin	M.D.	Senior Staff Fellow	LI, NEI
	François Roberge	M.D.	Visiting Scientist	LI, NEI
	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI

COOPERATING UNITS *(if any)*

Department of Critical Care Medicine, Clinical Center (Henry Masur, M.D.); Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases (H. Clifford Lane, M.D.); Pediatric Branch, National Cancer Institute (Phil A. Pizzo, M.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients suffering from AIDS (acquired immune deficiency syndrome) are at risk of developing significant ocular problems, either as a result of HIV (human immunodeficiency virus) itself or as a result of opportunistic infection. Some of these problems can lead to blindness if left untreated. Among the many pathogens that can lead to blindness, cytomegalovirus (CMV) is by far the most common. In FY 1993 the two major emphases have been (1) CMV retinitis detection and therapy and (2) pediatric AIDS. All currently available drugs are virostatic. Early detection is the most effective means of ensuring that patients will preserve long-term vision. We have continued to evaluate the usefulness of a laser photometric device to help screen patients for the presence of ocular inflammation and/or CMV retinitis. We also have looked at the use of alternative methods of followup using tangent screens and Amsler grids to help determine early recurrences. We have initiated a study using an implantable slow-release device for ganciclovir. This study is still in its early stages. Due to the sustained nature of the release, it is possible that this approach will lead to prolonged remissions, as compared to standard therapy.

In FY 1993 we continued to evaluate the incidence of ocular infection in about 220 children with AIDS. The incidence of complications is rare, which has prompted us to reduce the frequency of followups to every 6 months instead of every 4 months. However, it is still important that parents or guardians monitor their AIDS-affected children for symptoms and signs of visual loss.

Project Description

Additional Personnel

Susan Mellow R.N.

Clinical Protocol Number

90-EI-208

Objectives

This project's primary objective is to develop methods of identifying and treating known ocular complications of HIV (human immunodeficiency virus) infection in such a way as to prevent significant loss of vision. The second, equally important objective is the identification of new manifestations of ocular involvement from the AIDS (acquired immune deficiency syndrome) virus itself and related opportunistic ocular infections. The third objective is to recognize complications related to the therapeutic agents used or the mode of administration.

Methods

This project entails the clinical evaluation, diagnosis, and treatment of retinitis in AIDS patients. It also involves the development of novel methods of therapy for the various forms of retinitis observed. Study of pathologic tissue also is used to better understand the nature of the infectious processes.

Major Findings

In the past year our major effort has centered on the evaluation and treatment of cytomegalovirus (CMV) retinitis. CMV is a major vision-threatening infection found in patients with AIDS. Preservation of useful vision for a prolonged period of time requires early detection. In the present context, this can only be done by careful funduscopic examination by a trained eye care professional. In asymptomatic individuals, such exams are usually normal. Examinations in these patients are both time consuming and costly, with very few having any ocular lesions. The development of a screening device that could help to detect, within the eye, conditions predisposing to CMV retinitis and other disorders is highly desirable.

A device capable of detecting even minute fluctuations in the anterior chamber flare has been evaluated over the past 2 years. It consists of a low-intensity laser beam focused on the anterior chamber of the eye. Under normal circumstances, none of the incident light is reflected, as there is neither protein nor cells in the anterior chamber. In the presence of inflammation, part of the laser beam is reflected back through the cornea to a detector that measures the intensity of the reflected light in photons per millisecond. The intensity of this reflected light correlates directly with the intensity of inflammation in an affected eye. On average the test itself takes only 15 minutes for both eyes, and it is simple enough that it can be performed in a nonophthalmic clinic. We recently have evaluated data from 80 patients with and without CMV retinitis who were subjected to this test. We found that the technique was 100% sensitive in detecting patients with CMV retinitis when the cutoff count was 8.0 photons per millisecond; the corresponding specificity was 77%.

Early detection can lead to preservation of vision, but it also commits the patient to life-long intravenous (IV) therapy with anti-CMV drugs. A device recently has been developed that slowly releases ganciclovir directly into the eye. This alternative to systemic therapy may be particularly useful in patients who cannot tolerate IV infusions of antiviral drugs. We have developed a protocol to evaluate the safety and efficacy of the device in patients who have not been treated previously with an anti-CMV agent. The protocol is designed to compare the rates of progression between patients in whom therapy is delayed with the rates in patients implanted with a device that releases the drug over 8 months. Only patients with peripheral non-sight-threatening disease are eligible for the study. One important concern has been the lack of systemic coverage and its possible effects on patient survival. While the study is not designed to answer this question, patients will be followed carefully to determine whether this form of therapy has any adverse effect on their survival. Lack of systemic side effects from anti-CMV therapy and the patient's ability to stay on anti-HIV agents may in fact be of greater benefit to survival. So far, a dozen patients have been recruited out of a total of 35.

We have continued to follow about 200 children who developed AIDS by various means. We have been particularly struck by the lower incidence of CMV in this population, where the overall incidence is about 1.6%. However, in children who have low total T-cell counts (below 100/mm^3), the risk in-

creases to 16%. By following children every 6 months, we have been able to detect all cases of ocular involvement, provided that the children's parents or guardians are periodically screened for the presence of vision loss.

Significance to Biomedical Research and the Program of the Institute

The AIDS epidemic is a major public health concern. CMV retinitis remains the number one cause of blindness among patients infected with the AIDS virus. Early diagnosis is important because all drugs currently available are only virostatic and not virocidal; thus, some progression of the lesion is seen in more than 50% of patients, despite anti-CMV therapy. Inasmuch as most patients present late with well-established disease, a device able to screen and identify patients with early lesions is highly desirable. New therapeutic modalities that are cost-effective and reduce the incidence of progression or the development of resistant strains are necessary. Reports of strains resistant to gancyclovir are increasing in number.

The number of children infected with AIDS is on the rise. A good understanding of the epidemiology of AIDS in terms of ocular disease is highly desirable. We are therefore continuing to follow these children prospectively to identify the frequency and type(s) of ocular complications they are likely to develop.

Proposed Course

In the coming fiscal year we plan to evaluate further the laser photometer to determine possible ways of increasing the specificity of the device in detecting CMV retinitis. We will continue to evaluate the slow-release device in patients with newly diagnosed CMV retinitis. We also are planning to use new therapeutic agents for the treatment of CMV retinitis.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

de Smet MD: Ocular consequences of human immunodeficiency virus infection. *Ophthalmol Clin North Am* 6:117-126, 1993.

de Smet MD, Butler KM, Rubin BI, Whitcup SM, DeBarge LR, Martin DF, Pizzo PA, Nussenblatt RB: The ocular complications of HIV in the pediatric population, in Dernouchamps JP, Verougstraete C, Capsers-Velu L, Tassignon MJ (eds): *Recent Advances in Uveitis, Proceedings of the Third International Symposium on Uveitis.* New York, Kugler Publications, 1993, pp 315-319.

Muccioli M, Belfort R, Podgor M, Sampaio P, Hayashi S, Neves R, Lottemberg C, Kim MK, de Smet M, Nussenblatt RB: The diagnosis of intraocular inflammation and CMV retinitis in HIV infected patients by laser flare photometry. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1110, 1993.

Polis MA, de Smet MD, Baird BF, Mellow S, Falloon J, Davey RT, Kovacs JA, Palestine AG, Nussenblatt RB, Masur H, Lane HC: Increased survival of a cohort of patients with acquired immunodeficiency syndrome and cytomegalovirus retinitis who received sodium phosphonoformate (foscarnet). *Am J Med* 94:175-180, 1993.

Whitcup SM, Fenton RM, Pluda JM, de Smet MD, Nussenblatt RB, Chan C-C: Pneumocystis carinii and Mycobacterium avium-intracellulare infection of the choroid. *Retina* 12:331-335, 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00266-04 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Immune Responses to S-Antigen

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
Others:	Igal Gery	Ph.D.	Head, Section on Experimental Immunology	LI, NEI
	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
	Margaret Cheung	M.D.	Senior Staff Fellow	LI, NEI
	François Roberge	M.D.	Visiting Scientist	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

One of the characteristics of S-antigen (S-Ag) and interphotoreceptor retinoid-binding protein (IRBP), which are retinal-specific antigens, is the ability to induce an intense autoimmune inflammation in the eyes of experimental animals when injected in the presence of an adjuvant. This disease; called experimental autoimmune uveitis (EAU), is critically dependent on T cells and antigen processing by appropriate antigen-presenting cells (APCs). Antigen processing, which occurs within the endocytic vesicles of the APCs, results in the production of small polypeptide subunits. These small polypeptides must then be protected from further degradation and transported to the cell surface where the interaction with the T cell takes place.

In FY 1993 we identified an intracellular protein that is capable of binding a major epitope of IRBP. It was identified first in rat B cells, which are good antigen-presenting cells. This binding protein appears to belong to the heat shock family of proteins, and its production appears to be upregulated under conditions of cellular stress. These stresses can be exogenous, such as heat, or they can result from more physiologic stresses, such as stimulation by lectins and bacterial cell wall products. This protein appears to be present not only in animal cells but also can be detected in human B cells.

Project Description

Additional Personnel

Sumeet Mainigi		Biologist, LI, NEI
Kalpana Rengerajan	Ph.D.	Biologist, LRCMB, NEI
Gerald J. Chader	Ph.D.	Chief, LRCMB, NEI
Barbara Wiggert	Ph.D.	Head, Section on Biochemistry, LRCMB, NEI

Clinical Protocol Numbers

84-EI-214
79-EI-49

Objectives

In Fiscal Year (FY) 1993 the study has concentrated mainly on identification and isolation of the intracellular binding proteins involved in preventing degradation of key immunopathogenic epitopes of interphotoreceptor retinoid-binding protein (IRBP) and S-antigen (S-Ag) within antigen-presenting cells of Lewis rats and humans.

Methods

Using B-cell lysate, we isolated the intracellular binding protein for R-15 (1169-1191), a major immunopathogenic epitope of IRBP, on a cyanogen bromide Sepharose 4b column. Because this particular protein is produced in very small amounts within cells, large numbers of B cells were needed. In rats, these were first obtained by panning, but to increase the yield and the purity of the cell population, we used a magnetic separator with paramagnetic beads. The efficiency of the separation was greatly increased by this approach, and it required much less time. This approach also ensured a highly viable population of cells, which appeared to be much more responsive to physiologic stressors than those obtained by panning.

R-15 also has been shown to cause an immune response in peripheral blood lymphocytes of some patients with uveitis; thus, an attempt was made to identify and isolate a similar intracellular binding protein in human antigen-presenting cells—namely, the B cell. To produce large quantities of B cells, we used the Epstein-Barr virus (EBV) to transform peripheral blood lymphocytes from patients and normal controls. EBV causes B cells to proliferate indefinitely. Although these cells are infected with a virus and are perpetually in a blastic phase, their antigen-presenting capabilities are not affected. Once transformed, these cells can be grown in very high numbers in a variety of cell growth systems. A combination of stirrer flasks, tissue culture flasks, and cell factories was found to be the most efficient way of growing these cells in sufficient numbers. Once the appropriate cell number was obtained, we subjected the cells to specific physiologic stressors to increase the production of the cellular binding protein.

Major Findings

Our previous studies in the Lewis rat have shown that several fragments of S-Ag are able to induce a strong immune response when tested *in vitro*. These fragments are normally produced by endocytic enzyme degradation of a parent protein such as S-Ag or IRBP. Partially degraded fragments must then be protected from further degradation and transported to the cell surface, where they can associate with class II antigens. Recently it has been suggested that proteins belonging to the heat shock family of proteins might play a role in preventing enzymatic degradation and in carrying antigens to the cell surface. In FY 1993 we showed that antigen-presenting cells contain a protein that is able to bind to the immunodominant determinant of IRBP (sequence 1169-1191). This peptide-binding protein has a molecular weight similar to that of other heat shock proteins (HSP). By Western blot, it stains positively to monoclonal antibodies directed against the constitutive and inducible forms of HSP70. Binding does not appear to occur to all peptide fragments of IRBP, as shown in some preliminary experiments using Sepharose columns activated with different epitopes of IRBP. We also have isolated a similar protein from transformed human B cells. This protein has the same molecular weight and staining characteristics as the protein isolated from the rat cells. In both cell types, the protein is produced in larger amounts when the cell is activated by exogenous stress (heat) or by agents such as lipopolysaccharide. In addition to the 70-kD protein, there appears to be a secondary peak at 40-kD that is nearly always present. Its exact nature and role remain to be determined.

Significance to Biomedical Research and the Program of the Institute

Intracellular processing is a crucial step in the generation of an immune response. These studies suggest that certain intracellular proteins may play a determining role in the selection of the peptidic determinants that are ultimately presented at the cell surface. In addition, exogenous and endogenous factors appear to regulate the synthesis of these proteins. Identification of these intracellular proteins and the mechanisms that regulate their synthesis may give us further insights on the mechanisms of antigen presentation and possibly a means of regulating aberrant antigen presentation.

Proposed Course

In the coming year the main emphasis will be on further characterization of the intracellular binding protein. We will attempt to determine the binding characteristics of the protein and to determine the factors that can enhance its synthesis.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

de Kozak Y, Mirshahi M, Stiemer R, de Smet M, Frank R, Faure JP: Modulation of S-antigen induced EAU by neonatal injection of peptides from S-Ag or TNF-α or by anti-idiotypic antibody. *Exp Eye Res* 55(suppl 1):S84, 1992.

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de Smet MD, Mainigi S, Nussenblatt RB: Immunogenicity and immunopathogenicity of peptide determinants of human S-Ag in various rat strains. *Invest Ophthalmol Vis Sci* 34(4)(suppl): 1143, 1993.

Rengarajan K, de Smet MD, Chader GJ, Wiggert B: B cells in Behçet patients contain a heat shock protein that binds to a fragment of IRBP. Clinical Immunology Society, Denver, CO, 1993, p 72A.

Rengarajan K, de Smet MD, Chader GJ, Wiggert B: Identification of a heat shock protein that binds to a peptide causing autoimmune uveitis. Keystone Meeting on Molecular Chaperones: Function in Protein Folding and Cellular Metabolism. Keystone, CO, October 1992.

Rengarajan K, de Smet MD, Chader GJ, Wiggert B: Identification of a heat shock protein that binds to peptide 1169-1191 of IRBP causing autoimmune uveitis. *Invest Ophthalmol Vis Sci* 34(4)(suppl): 1482, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00276-02 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Surgical Management of Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI
Others:	François Roberge	M.D.	Visiting Scientist	LI, NEI
	Margaret Cheung	M.D.	Senior Staff Fellow	LI, NEI
	Scott Whitcup	M.D.	Senior Staff Fellow	LI, NEI
	David Parks	M.D.	Senior Staff Fellow	LI, NEI
	Dan Martin	M.D.	Senior Staff Fellow	LI, NEI
	David Callanan	M.D.	Senior Staff Fellow	LI, NEI
	Naofumi Hikita	M.D.	Visiting Associate	LI, NEI
	Ray DeBarge	M.D.	Senior Staff Fellow	LI, NEI
	Richard Fenton	M.D.	Senior Staff Fellow	LI, NEI

COOPERATING UNITS *(if any)*

Clinical Oncology Program, Medicine Branch, National Cancer Institute (Robert Wittes, M.D.)

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.75	1.75	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with uveitis often develop ocular complications that require surgery to prevent permanent loss of vision. Surgery in these patients has been particularly challenging because the surgery itself can induce severe inflammation. The exact timing of the surgery and the choice of postoperative immunosuppressive therapy given to the patient often determine the outcome. Proper handling of the specimen is essential, particularly in cases of intraocular lymphoma in which the lymphoma cells are particularly fragile. In patients with recurrent disease, doing an air-fluid exchange can provide the necessary cells to make a diagnosis. Glaucoma remains an important complication in patients with uveitis. In all cases, standard trabeculectomies stop functioning after a few months. We are continuing to compare the use of 5-FU and Molteno implants in patients with uveitis and glaucoma who require surgery. We have enrolled 12 patients in the study.

The use of intraocular lenses following cataract extraction in patients with uveitis is being addressed in a randomized double-masked study to compare modified intraocular lenses with standard lenses in patients with uveitis that has been under control for at least 3 months. So far three patients have been enrolled in the study, and no significant complications have been seen.

In experimental models, we evaluated the effect of different methods of immunomodulation on graft rejection. In a corneal graft rejection model in rats, we evaluated the kinetics of inflammatory cell infiltration into the graft with and without FK 506 treatement. We also began to evaluate the effect of feeding class I and class II antigens on the rejection rate of corneal grafts. We also have initiated a study of retinal pigment epithelial cell transplantation in the Lewis rat. The immunohistochemical characteristics of the graft were studied for several weeks. Results thus far indicate that rejection occurs at the same rate as in any other tissue.

Project Description

Additional Personnel

Igal Gery	Ph.D.	Head, Section on Experimental Immunology, LI, NEI
Chi-Chao Chan	M.D.	Head, Section on Immunopathology, LI, NEI
Susan Mellow	R.N.	Nurse, LI, NEI
Susan Whitcher		Psychologist, LI, NEI

Clinical Protocol Numbers

79-EI-49
87-EI-104
92-EI-157

Objectives

The objectives of this project are as follows: (1) to develop rational surgical approaches for patients with intraocular inflammation, because appropriate surgical modalities are needed to properly manage the complications that arise with chronic intraocular inflammation; (2) to devise rational methods for sampling intraocular tissues and to develop the methodology needed to obtain clinically useful information from limited tissue samples; (3) to test new methods of suppressing graft rejection in animal models; and (4) to determine the immunology of graft rejection in the subretinal space.

Methods

Patients who have developed ocular complications as a result of ocular inflammation and who require surgery and patients for whom an appropriate diagnosis can only be made with surgery are eligible for one of the patient protocols described above. Patients with vitritis and retinitis of unknown etiology in whom a nonspecific trial of immunosuppression is contraindicated may, according to the protocol, undergo vitrectomy and chorioretinal or endoretinal biopsy to obtain a diagnosis. The tissue specimen is partitioned for microbiology, electron microscopy, immunohistochemistry, and polymerase chain reaction.

Patients with a suspected intraocular lymphoma undergo an intraocular lymphoma workup with appropriate computed tomography or magnetic resonance imaging scans and lumbar punctures. If these are negative, a vitrectomy is performed and the cells are studied by immunohistochemistry. Patients who have intraocular lymphoma are entered in a central nervous system (CNS) lymphoma protocol and followed prospectively.

Patients with glaucoma and uveitis are entered in a double-masked trial of either trabeculectomy with 5-fluorouracil or Molteno implant. They are then followed prospectively to determine the degree of postoperative inflammation and to determine how effective the procedure is over time.

For patients with cataracts and uveitis under good control and minimal intraocular inflammation, the protocol calls for a cataract extraction and randomization to a standard intraocular lens or a modified lens with a heparin coating. Patients are then monitored postoperatively for the appearance of inflammation via laser cell flare meter. They also are monitored for the appearance of cellular deposits on the lens surface.

In animals, we test the efficacy of oral feeding of class I and class II antigens in preventing corneal graft rejection. We also evaluate the kinetics of inflammatory cell infiltration in corneal grafts that are treated with a placebo or FK 506. This model uses heterotopic grafts taken from Fisher rats and sewn into Lewis rats. To evaluate the rejection characteristics of retinal pigment epithelial (RPE) cells under conditions that would favor rejection, human RPE cells are implanted into the subretinal space of Lewis rats using a transscleral approach. Animals are serially sacrificed at preset times to determine the severity of rejection and the type of cell infiltration occurring within the grafted tissue. Standard immunohistochemistry for avidin-biotin-peroxidase reactions is used in these experiments. Finally, using the endotoxin-induced uveitis model, we are testing a method of measuring, in a noninvasive way, the inflammation present in the anterior chamber of rats. The device used is a laser photometer that already is being used in patients to monitor anterior chamber inflammation. Measurements of anterior chamber inflammation made with the device are correlated with measurements of the protein in the anterior chamber.

Major Findings

In Fiscal Year 1993 we performed several diagnostic vitrectomies for intraocular lymphoma. This particular lymphoma, a subtype of CNS lymphomas, is on

the rise. There are three times more CNS lymphomas being diagnosed today than 10 years ago. Prior to performing a vitrectomy, we perform a complete workup, including an MRI brain scan and lumbar puncture on each patient. Several patients were referred to us after unsuccessful attempts to diagnose the tumors elsewhere. Invariably, after reviewing the charts, we found that the specimens had been poorly processed or had been allowed to sit for too long. We have found that it is imperative to bring a specimen to the pathology laboratory while the vitrectomy is under way to ensure adequate cell viability. In a patient who has had a previous vitrectomy and in whom there are still cells floating in the vitreal cavity, a simple air/fluid exchange may be all that is necessary to make the diagnosis.

We have found that pretreatment with pulse methylprednisolone is an effective way of decreasing preoperative inflammation. This has been particularly useful in cases in which it has been necessary to perform a vitrectomy while there was still evidence of inflammation. In patients undergoing the Molteno implant, interim analysis seems to suggest that Molteno implants maintain a lower pressure for a longer period of time. Most trabeculectomies fail by about 18 months, while Moltenos are still functioning after 24 months. We also have found that in all postoperative cases, the use of topical nonsteroidal agents such as diclofinac significantly reduce the amount of inflammation. This is particularly visible in glaucoma patients. No data analysis is yet available on the intraocular lens trial, which has just begun.

In the animal studies, we were able to demonstrate that topical drops of FK 506 are an effective means of stopping corneal graft rejection. FK 506 has a predominant effect on T cells, inhibiting both the activation of these cells and their recruitment into the transplanted tissue. It appears to down-regulate the expression of both class I and class II antigens as well as adhesion molecules in the transplanted cornea. Using these drops, we are now looking at the influx of cells into the graft tissue to determine the early events involved in graft rejection. Preliminary studies of feeding lymphocytes of donor animals to recipients prior to corneal grafting are showing promising results. There appears to be a delay in corneal graft rejection, but the effect is not yet statistically significant.

Studies on the kinetics of RPE rejection in the subretinal space of Lewis rats reveal that, when human RPE cells are used, graft rejection occurs within 14 days. This is the same rate of rejection observed for other tissue grafts. The infiltrating cell population is mixed, containing both T and B cells. This is the first good demonstration of graft rejection in RPE transplantation. Prior to these experiments, several claims had been made that graft rejection did not occur. Studies using the laser photometer have shown that it is possible to take measurements of anterior chamber flare from rat eyes. The major advantages of this technique are that it can be performed on live, anesthetized animals; measurements can be repeated over time; and the measurements are given as numerical values, making data analysis much easier.

Significance to Biomedical Research and the Program of the Institute

Uveitis is the cause of 10% of visual impairment in the United States. Ocular complications that require surgery for correction are common in these patients, despite adequate immunosuppression. Developing appropriate surgical modalities of treatment is thus an important endeavor. Similarly, conditions exist in which appropriate therapy can only be given once the proper diagnosis has been made from intraocular tissue. Developing the means of obtaining a minimal amount of tissue and properly processing it is thus of major significance.

Proposed Course

This study will continue to investigate methods of surgically managing patients with uveitis. Patient enrollment continues. In the animal models, we will continue to study new methods of modulating graft rejection. We also will proceed with our study of the immunology of RPE transplantation and factors that may influence rejection, such as the method of graft insertion. A transvitreal approach may prove to be less inflammatory.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

DeBarge LR, Fenton R, Nussenblatt RB, de Smet MD: Quantitative determination of the flare in

the anterior chamber of the rat by a noninvasive method. *Invest Ophthalmol Vis Sci* 34(4)(suppl): 1481, 1993.

Fenton RM, Rubin BI, de Smet MD, Whitcup SW, Nussenblatt RB: A prospective study of 5-FU trabeculectomy vs. single plate Molteno implant in patients with panuveitis complicated by glaucoma refractory to prior therapy. *Invest Ophthalmol Vis Sci* 34(4)(suppl):897, 1993.

Hikita N, Lopez JS, Chan C-C, Mochizuki M, Nussenblatt RB, de Smet MD: Effect of topical FK 506 on the rejection of corneal allograft in the Lewis rat. 2nd International Symposium on Ocular Inflammation, Jerusalem, Israel, Aug 30–Sep 3, 1992.

Martin DF, Chan C-C, de Smet MD, Palestine AG, Davis JL, Whitcup SW, Burnier MN, Nussenblatt RB: The role of chorioretinal biopsy in the management of posterior uveitis. *Ophthalmology* 100:705-714, 1993.

Parks DJ, Hikita N, Nagineni C, Hooks JJ, Chan C-C, Nussenblatt RB, de Smet MD: Immunohistochemistry of xenogeneic RPE transplants in the rat: A model for graft rejection. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1095, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00115-15 LI

PERIOD COVERED: October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Cyclosporine Therapy in Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert B. Nussenblatt	M.D.	Scientific Director	NEI
Others:	Marc D. de Smet	M.D.	Senior Staff Fellow	LI, NEI
	Scott Whitcup	M.D.	Senior Staff Fellow	LI, NEI
	Chi-Chao Chan	M.D.	Head, Section on Immunopathology	LI, NEI
	Richard Fenton	M.D.	Special Volunteer	LI, NEI
	Dan Martin	M.D.	Senior Staff Fellow	LI, NEI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Laboratory of Immunology

SECTION: Section on Immunoregulation

INSTITUTE AND LOCATION: NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	0.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To test its efficacy in the treatment of uveitis, cyclosporine—an endecapeptide fungal product with specific anti-T-cell characteristics—is being administered to patients with sight-threatening ocular inflammatory disease of noninfectious origin who have failed on either corticosteroid or cytotoxic agent therapy. Within the context of these ongoing studies, the combined use of cyclosporine A and ketaconazole has been tested in a randomized masked study of a small group of patients whose uveitis was well controlled with cyclosporine. The combination allowed a significant reduction in the dose of cyclosporine needed to control the disease. In some instances the dose could be reduced by as much as 90%. No significant increase in side effects was noted. A phase I/II randomized trial using cyclosporine A and cyclosporine G has ended. There is a definite trend showing that combined use of a cyclosporine and low-to-moderate steroid doses are efficacious in preventing the progression of uveitis. An effective dose of cyclosporine appears to be around 5 mg/kg. At this dosage, toxicity has been reduced for up to 12 months of followup. Cyclosporine G was more effective than cyclosporine A in treating cystoid macular edema.

Project Description

Additional Personnel

Barry Grubbs Biologist, LI, NEI

Clinical Protocol Number

81-EI-33

Objectives

Cyclosporine, an endecapeptide obtained from fungi, has been shown to have specific anti-T-cell activity (*Transplant Proc* 12:234, 1980). We have reported cyclosporine's exceptional effectiveness in preventing the induction of S-antigen (S-Ag) autoimmune uveitis in rats, as well as in inhibiting the disease once immunization has occurred (*J Clin Invest* 67:1228, 1981). The goal of this study is to test cyclosporine A (CsA) versus cyclosporine G (CsG) to test their efficacy in treating patients with bilateral sight-threatening posterior uveitis of an autoimmune nature.

Methods

Patients 18 years or older, of either sex (females not pregnant) who have not done well on more conventional medical therapy have been admitted to this study. All patients must have bilateral sight-threatening uveitis of noninfectious etiology that was not satisfactorily controlled by either corticosteroid or cytotoxic agent therapy. Lymphocyte cultures are prepared, and the immune cells are tested against various crude ocular extracts, as well as purified human S-Ag, to assess evidence of cellular immune memory, which is considered to be the *in vitro* equivalent of the anamnestic response *in vivo*. Patients chosen are treated with CsA or a new analog called CsG in a phase I/II trial to evaluate the safety and activity of CsG versus CsA. During this period, the patients' clinical and immunologic courses are closely monitored. Specific attention is given to renal function change, a frequent side effect. Patients who need to continue CsA for over 1 year because of their ocular disease may be asked to undergo renal biopsy for evaluation of the reversible and irreversible components to CsA renal toxicity. Some patients entered on previous CsA studies still followed in the eye clinic will continue to be monitored for their renal function to determine how and when cyclosporine dosage can safely be tapered.

Major Findings

CsA has been effective in the treatment of some cases of posterior uveitis. Decreased inflammatory activity and improved visual acuity was seen in most patients treated to date. The particular responsiveness to this agent by patients with the ocular manifestations of Behçet's disease has been corroborated by a masked randomized trial performed in Japan. The improvement in the clinical condition was supported by a concomitant improvement in electrophysiologic test results, particularly in contrast sensitivity.

Patients treated with CsA had no abnormalities of natural killer cell activity before the initiation of therapy, nor was any noted afterward. CsA significantly decreased skin test responsiveness but did not alter lymphocyte proliferation or antibody production in patients. Renal toxicity has been noted in some patients on long-term therapy, necessitating the addition of systemic corticosteroids and a decrease in CsA dosage. At 3 months approximately 78% of the patients entering this open study were considered therapeutic successes, while 62% were considered successes at 1 year.

Seventeen patients treated long term with CsA underwent renal biopsy. These biopsy specimens were read in a masked fashion by a group of renal disease specialists who compared these biopsies to those from age-matched controls. An irreversible component of CsA toxicity could be identified: in the main, renal tubular atrophy accompanied by interstitial fibrosis. The majority of the individuals' biopsies had normal serum creatinine values, but a correlation could be made between the alterations noted and previous serum creatinine elevations for some period of time. The cyclosporine A/G trial has shown that the two cyclosporines have overall equal value in treating uveitis. However, CsG was more effective than CsA in reducing cystoid macular edema, particularly at lower dosages.

Significance to Biomedical Research and the Program of the Institute

Uveitis is one of the most frustrating problems in all of ophthalmology. Present modes of therapy for patients with severe ocular inflammatory disease are inadequate and nonspecific. CsA appears effective in treating posterior uveitis of noninfectious etiology. This is the first new agent in decades to be found

useful in treating the severe form of this condition; therefore, it is important that the optimum therapeutic schedule be developed. Newer therapeutic strategies have already begun.

Proposed Course

Newer studies to look at various cyclosporine combinations will continue.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

de Smet MD, Nussenblatt RB: Clinical use of cyclosporine in ocular disease. *Int Ophthalmol Clin* 33(4):31-45, 1993.

Nussenblatt RB, de Smet MD, Rubin B, Freidlin V, Whitcup SM, Davis J, et al: A masked randomized, dose-response study between cyclosporine A and G in the treatment of sight-threatening uveitis of noninfectious origin. *Am J Ophthalmol* 115:583-591, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00278-02 LI

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 charecters or less. Title must fit on one lina betwaen the borders.)*

Oral Administration of Antigen and the Ocular Immune Response

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert B. Nussenblatt	M.D.	Scientific Director	LI, NEI
Others:	Igal Gery	Ph.D.	Head, Section on Experimental Immunology	LI, NEI
	Susan Whitcher	M.S.	Clinical Protocol Assistant	LI, NEI
	Marc D. de Smet	M.D.	Visiting Scientist	LI, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunology

SECTION

Section on Immunoregulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.8	0.3	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

The effect of oral administration of various antigens on the ocular immune response has been tested in the animal model for a severe intraocular inflammatory disease, experimental autoimmune uveioretinitis, which is induced by both retinal S-antigen (S-Ag) and interphotoreceptor retinoid-binding protein (IRBP). Oral tolerance could be induced by repeatedly feeding rats S-Ag. A putative suppressor cell that was CD8 positive could be isolated from the spleen of such animals and transferred to other animals to induce a similar toleragenic effect. In addition, the role of the spleen was confirmed in ongoing animal experiments. A randomized, masked trial to evaluate the usefulness of S-Ag feeding in patients with intraocular inflammatory diseases has been put together. A pilot study performed in two patients showed the induction of such tolerance.

Project Description

Objectives

Exploring means of immunomodulation has been a major role in this laboratory. While extensive experimentation has used various immunosuppressive agents, there also has been a major thrust in attempts to use other modes of immunosuppression. The goal of this series of experiments, both in animals and in humans, is to test the efficacy of oral tolerance with uveitogenic antigens in the treatment of animals induced with experimental autoimmune uveitis and in patients with bilateral sight-threatening posterior and intermediate uveitis of an autoimmune nature.

Methods

Six- to 10-week-old Lewis rats of either sex are used for these experiments. Animals are fed various antigens both before and after the induction of experimental uveioretinitis. The antigens include whole molecules such as the retinal S-antigen (S-Ag) and interphotoreceptor retinoid-binding protein (IRBP), as well as their fragments. In a subset of experiments, some animals also undergo splenectomy before the initiation of the experiments; other animals receive sham procedures. We are attempting to evaluate the clinical course of the disease and corroborate the clinical observations with histopathology at various points after initiation of the experiments. The goal is to evaluate the role of the spleen, as well as the role of various fragments, in the ability to induce this toleragenic state.

In the studies performed with patients, individuals who have bilateral uveitis of a noninfectious cause and are 18 years or older (either sex) are considered for the study. In addition, their lymphocytes must demonstrate an *in vitro* proliferative response to the retinal S-Ag. The patients also need to be on systemic immunosuppressive therapy, whether it be corticosteroids, cytotoxic agents, or cyclosporine. The goal of this study is to determine whether the addition of oral feeding of retinal antigens will induce a toleragenic state in individuals who need high amounts of immunosuppressive therapy to control their disease.

This study is performed in a randomized, double-masked fashion in which some patients receive S-Ag, other patients receive a retinal mixture containing several antigens, and still other patients receive placebo. The intent is to reduce the amount of immunosuppressive therapy that the patients are taking. We hope that a toleragenic state can be induced by feeding these antigens.

Major Findings

In the animal study, the spleen appears to play an important role in the induction of oral tolerance of S-Ag. In addition, the spleen is essential for adoptive transfer of tolerance by splenocytes from donors fed S-Ag. Thus, it would be logical to assume that the spleen acts as a site for induction and/or amplification of cells with suppressive activity.

The pilot study demonstrated that, at least in two patients, a toleragenic state can be induced by feeding antigen at the dosages planned for this study. One patient with par planitis and one with Behçet's disease have been able either to come off medication completely or to be reduced to exceptionally low dosages.

Significance to Biomedical Research and the Program of the Institute

Uveitis is one of the most frustrating problems in all of ophthalmology. The present modes of therapy for patients with severe ocular inflammatory disease all have limitations, in particular because of their secondary effects. By identifying patients with an immune response to the retinal S-Ag, we will be able to induce an immunosuppressive state without the use of pharmacologic agents. Furthermore, the induced tolerance would be antigen specific.

Proposed Course

The randomized study will begin shortly.

NEI Research Program

Retinal and Choroidal Diseases—Inflammatory Disorders

Publications

Nussenblatt RB, de Smet MD, Weiner HL, Gery I: The treatment of the ocular complications of Behçet's disease with oral tolerization, in Wechsler B, Godeau P (eds): *Sixth International Conference on Behçet's Disease.* New York: Excerpta Medica, 1993.

Weiner HL, Miller A, Khoury SJ, Zhang ZJ, AL-Sabbagh A, Brod SA, Lider O, Higgins P, Sobel R, Matsui M, Sayegh M, Carpenter C, Eisenbarth G, Nussenblatt RB, Hafler DA: Suppression of organ-specific autoimmune diseases by oral administration of autoantigens. *Progress in Immunology VII. Eight International Congress of Immunology*, Budapest, 1992.

Laboratory of Mechanisms of Ocular Diseases

Report of the Acting Chief, Laboratory of Mechanisms of Ocular Diseases

J. Samuel Zigler, Jr., Ph.D.

Investigators in the Laboratory of Mechanisms of Ocular Diseases (LMOD) have continued to conduct studies on a broad range of topics relating to the biology of various tissues in the normal eye and the molecular mechanisms responsible for certain ocular diseases. The major emphasis has been on cataract and the various ocular complications of diabetes.

Section on Cataract

Dr. Deborah Carper and her colleagues have concentrated their efforts on the role of aldose reductase, which produces polyols, in causing diabetic complications and on the possible effect of sorbitol dehydrogenase, which metabolizes polyols, in protecting against such pathologies. Specifically, site-directed mutagenesis studies have demonstrated that the histidine at position 110 of aldose reductase is critical for catalytic activity. Also, a study has been instituted on a family with congenital cataracts, whose members have a probable genetic defect in the sorbitol dehydrogenase gene.

Dr. Donita Garland's group has made major advances in its collaborative study on the protein composition of normal human lens and cataracts. The addition of a scanner capable of quantifying the complex images obtained by two-dimensional electrophoresis and software with which to compare and analyze the data provides the tools necessary to address the important questions raised by this investigation. In addition, this group is investigating the effects of metals, including copper, iron, and zinc, on the lens crystallins and has found that both oxidation and aggregation are induced by such exposure *in vitro*.

Dr. Fielding Hejtmancik and his group are studying structure/function relationships of β-crystallins and doing gene-mapping studies on a variety of genetic diseases with ocular findings. One such disease is Usher's syndrome type I, for which two independent genes have been mapped on chromosome 2. One of these genes, which causes Usher's syndrome in the Acadian population, has been localized within a 6cM portion of the chromosome.

Dr. Paul Russell's group has concentrated its efforts on the biology of the lens epithelium and on development of lens organ culture techniques. Studies on lens epithelium have included analysis of protective mechanisms induced by various types of stress and analysis of the process whereby lens epithelial cells differentiate into fibers. These studies include tissue culture approaches as well as analyses of the epithelial layer from intact lenses. A novel method has been developed to assess the integrity of lenses in organ culture. This technique provides quality control information which allows the researcher to reject imperfect lenses before committing them into experiments.

Dr. Samuel Zigler's group also has been working with the lens organ culture system, using it as a means of screening potential anticataract drugs. This group also is investigating the functions of lens crystallins, in particular the role of α-crystallin as a molecular chaperone. Definitive proof of noncovalent complex formation between α-crystallin and the early non-native forms of denaturing proteins has been obtained, as has evidence for marked differences in the protection of apo- and holo- forms of some enzymes.

Section on Pathophysiology

Dr. W. Gerald Robison, Jr., has continued to refine and better characterize the rat model for diabetic retinopathy. Multiple angiopathies were present in the retinas of these rats following 24 months of galactose feeding. In contrast, galactose-fed animals given an aldose reductase inhibitor did not develop such pathologies. The data support the hypothesis that aldose reductase is the primary player in the formation of retinopathy in this model.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00201-09 LMOD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure and Expression of Polyol Pathway Enzymes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Deborah Carper	Ph.D.	Biologist	LMOD, NEI
Others:	Susan Old	Ph.D.	Staff Fellow	LMOD, NEI
	Takeshi Iwata	Ph.D.	Visiting Associate	LMOD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In diabetes, the accumulation of sorbitol is believed to be a key factor in initiating cataract, retinopathy, and neuropathy. We are interested in controlling the accumulation of sorbitol by regulating the action of the two enzymes of the sorbitol pathway: (1) aldose reductase (AR), which reduces glucose to sorbitol, and (2) sorbitol dehydrogenase (SDH), which oxidizes sorbitol to fructose. Our aim is to design innovative methods to inhibit the action of AR or increase SDH, with the purpose of reducing sorbitol accumulation in diabetic tissues.

Site-directed mutagenesis of AR has been a major priority of our laboratory. We have made amino acid substitutions in the rat and human AR and determined that some of these changes affect the kinetics of the protein with its substrate. For example, when histidine at position 110 was changed to glutamine, the activity of AR was reduced dramatically. The H110Q mutant protein showed very little activity with glyceraldehyde (1% of normal) and no activity with *p*-nitrobenzaldehyde. Other histidine substitutions did not acutely alter the kinetics of AR, supporting the finding that H110 plays an essential role in catalysis. These structure/function studies should help define the active site and locate the target areas of the current AR inhibitors.

Another strategy to control sorbitol accumulation is to regulate SDH. We have determined the primary sequence of human SDH and characterized part of the SDH gene. Molecular genetic studies also are under way to test for an SDH genetic defect in a family presenting with congenital cataracts and lowered SDH enzyme activity. By evaluating the expression of SDH at the gene level, we may be able to evaluate its role in sorbitol accumulation in diabetes and other genetic diseases.

Project Description

Objectives

The objective of this project is to study regulation of the enzymes of the polyol pathway.

Methods

The methods employed include molecular biology, protein chemistry, and cell biology techniques.

Major Findings

Structure/function studies.—Mutant forms of the aldose reductase (AR) protein were synthesized using the polymerase chain reaction. Sequencing verified the amino acid substitution. The mutant proteins were expressed in bacteria, purified on three columns using different biochemical characteristics of the protein, then tested for enzyme activity. Of the six histidines we mutated, histidine at position 110 was discovered to play an essential role in enzyme catalysis. When histidine 110 was changed to glutamine, AR activity was reduced to only 1% of the normal. The K_m for glyceraldehyde changed from 0.12 mM for the normal to 12.5 mM for the H110 mutant. No reductase activity was observed for H110Q using *p*-nitrobenzaldehyde as a substrate. Ultraviolet circular dichoism and NADPH fluorescence quenching indicated that H110Q was not substantially altered in structure or in its ability to bind NADPH. Because of its location in the active site pocket, histidine 110 has been proposed to be a hydrogen donor in the catalytic mechanism of AR. From these findings, using site-directed mutagenesis, we conclude that H110 plays a critical role in the catalytic mechanism of AR, although further studies will be needed to determine the exact nature of its action.

Expression of human AR in transgenic mice.—A cDNA that encodes human AR was ligated with a ζ-crystallin promoter. The construct was injected into mice. Several mice were found to carry the transgene and were bred to produce separate F_1 generations. Evaluation of the presence of the enzyme and its polyol product are now under way. Expression of human AR in transgenic mice will facilitate *in vivo* drug design studies.

Characterization of sorbitol dehydrogenase (SDH) in a family with congenital cataracts.—We have obtained and sequenced over 50% of the gene for human SDH. Previously we determined the complete coding sequence of the protein. With this information we have begun studies to determine a possible genetic defect in a family reported to have reduced levels of SDH and congenital cataracts. Our preliminary nucleotide-sequencing data have indicated a difference between this family and normal controls.

Significance to Biomedical Research and the Program of the Institute

AR has been implicated in diabetic cataracts, retinopathy, and neuropathy. Side effects and lack of efficacy of AR inhibitors in diabetic clinical trials have emphasized the need for innovative approaches to AR inhibition. Our research is a rational approach to designing new types of inhibitors by characterizing the structure/function aspects of the protein and evaluating the signals that regulate this enzyme. In addition, we feel that by understanding the regulation of SDH—the other enzyme of the polyol pathway—we may be able to modulate more fully the accumulation of sorbitol in diabetes.

Proposed Course

The project will continue via site-directed mutagenesis of AR protein to localize the critical amino acid residues in the active and inhibitor binding sites. We will complete the structure of SDH and analyze the gene in a family with congenital cataracts.

NEI Research Program

Cataract—Molecular Genetics

Publications

Bateman JB, Kojis T, Diep A, Klisak I, Heinzmann BS, Carper D, Nishimura C, Mohandas T, Sparkes RS: Mapping of aldose reductase gene sequences to human chromosomes 1, 3, 7, 9, 11, 14 and 18. *Genomics*, in press.

Lin L-R, Carper D, Yokoyama T, Reddy V: Effect of hypertonicity on aldose reductase, alphaB-crystallin and organic osmolytes in retinal pigment epithelium. *Invest Ophthalmol Vis Sci* 34:2352-2359, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00189-10 LMOD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Oxidation of Proteins in Cataractogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Donita L. Garland	Ph.D.	Research Chemist	LMOD, NEI
Others:	Jose Jimenez	Ph.D.	Visiting Fellow	LMOD, NEI
	Lorenzo Merola	M.S.	Chemist	LMOD, NEI
	Kenichi Matsuno	Ph.D.	IRTA	LMOD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The proteins of the normal human lens and cataracts of various etiologies are being characterized. These studies include identifying the major protein species and the modified forms of these proteins, mapping the protein composition throughout the lens, and quantitating changes in the levels of these proteins in cataracts.

An enzyme that protects proteins against thiol-dependent oxidative inactivation has been identified in bovine, rat, and primate lens. The enzyme has been purified and identified by sequence analysis. Seventy percent of the amino acid sequence has been obtained.

The interaction of copper, iron, and zinc with a number of proteins, including lens crystallins, has been studied. All three metals alter the solubility of the lens crystallins and many of the other proteins studied. Copper and iron are metals generally thought to be involved in the metal-catalyzed oxidation of proteins. In addition, these studies show them capable of inducing aggregate formation.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

The immediate objectives of this project are (1) to identify and characterize the types of protein modifications found in cataracts of various etiologies, (2) to investigate the role of oxidation in the formation of these modifications, (3) to study the interaction between metals and crystallins and the effects of these interactions on crystallin solubility and aggregate formation, and (4) to characterize one of the enzymes that protect lens proteins against thiol-dependent metal-catalyzed oxidation.

Methods

Bovine, rat, and human tissues were used for these studies. After human lens material was obtained from donors' eyes via cataract surgery, we employed classical methods to purify bovine and rat lens proteins. Other methods used were standard procedures for studying proteins, including two-dimensional gel electrophoresis, high-pressure liquid chromatography, ultraviolet/visible spectroscopy, fluorescence, circular dichroism, electron spin resonance, amino acid analysis, and immunotechniques.

Major Findings

1. Copper, zinc, and iron, but not calcium, induced aggregate formation in bovine lens extracts and solutions of the crystallins. Aggregation, measured by light scattering, was time dependent, occurring at metal-to-protein ratios greater than 1.0 and varying, depending on the metal and protein. Zinc induced the aggregation of β- and α- but not γ-crystallin. Copper and zinc induced aggregation of a number of other proteins, but they had no effect on lysozyme and papain. One explanation for the lack of effect on these two proteins is that they are basic proteins. However, copper induced aggregation of γ-crystallin is also a basic protein.

The affinity of copper and zinc for these proteins is relatively low (K_d max is about 10^{-5} M). The addition of EDTA, DETAPAC, L-histidine, or L-cysteine prevented zinc- and copper-induced protein aggregation and caused complete disaggregation.

These studies suggest that histidine is the amino acid involved in the aggregation induced by zinc and possibly copper. The treatment of α- and β-crystallin and trypsin inhibitor with diethylpyrocarbonate prevented aggregation. Increasing the pH to 8.0 substantially increased the zinc-induced aggregation of α-crystallin but not that of β-crystallin. No pH-induced change in conformation of either protein was observed by fluorescence studies, suggesting the effect may have been on the pK of an amino acid. The presence of salt decreased the metal-induced aggregation of α- and β-crystallin. No metal-induced changes in secondary and tertiary structures of these proteins were observed by fluorescence and circular dichroism spectroscopy. The mechanism of metal-induced aggregation is not clear, but it is likely that it primarily involves cross-linking rather than conformation-induced protein-protein interaction.

The presence of small amounts of zinc in the buffer reduced the thermal stability of α-crystallin and hemoglobin.

Zinc concentrations greater than 20 μM induced cell membrane damage to rat lenses in culture, as measured by choline and rubidium uptake.

Atomic absorption analysis of bovine crystallins indicate the presence of zinc associated with α- and β-crystallin.

These studies clearly demonstrate that metals are known to be involved in oxidative damage and protection against oxidative damage bind crystallins. This interaction induces aggregate formation, a phenomenon that has been linked to cataractogenesis.

2. A protein that appears to function in detoxification of the products of thiol-dependent oxidation has been demonstrated in cow, monkey, and rat lens and human trabecular meshwork cells. The protein has been purified to apparent homogeneity, and about 70% of the amino acid sequence has been obtained. The enzyme has been identified from the protein sequence as one of the detoxification enzymes found in most cells. The absence of secondary sequences and the copurification of the antioxidant activity and the detoxification enzyme during two separate schemes strongly indicate that the antioxidant activity is associated with this detoxification enzyme, not with a contaminant in the preparation. There are no previous reports of the antioxidant activity of this enzyme.

3. Analysis of the proteins in human cataract specimens by two-dimensional gel electrophoresis has continued and the techniques have been optimized. Preparation procedures to facilitate analysis of aspirated lens material (primarily outer cortex

obtained during extracapsular cataract surgery) have been established. These procedures allow us to determine the relative amount of the major proteins present and the oxidation state of these proteins in the cataract.

We have identified alterations in protein patterns and are doing quantitative analyses of the changes. Correlations between the altered patterns and cataract etiologies are being sought.

4. We have developed capillary gel chromatography procedures that allow the separation and accurate quantitation of sorbitol and galactitol.

Significance to Biomedical Research and the Program of the Institute

Oxidative processes have long been considered a major contributing factor in senile cataracts. Metal-catalyzed oxidation of the crystallins leads to protein modifications that mimic those seen in aging and senile cataracts and in brunescent lenses. These studies continue to demonstrate the potential for metal involvement in cataract formation. Not only do these metals facilitate oxidative modification of the proteins, they also can induce protein aggregation.

Understanding the lens' mechanisms of protecting itself against oxidative damage is important for developing interventions. These studies demonstrate the presence in the lens of an enzyme that protects against thiol-dependent oxidation. This activity is associated with a detoxification enzyme present in most cells and is induced under oxidative stress.

The importance of characterizing the proteins in the human lens—normal and cataractous—is obvious. It will give us a wealth of information on aging processes, mechanisms involved in cataractogenesis, and metabolic processes in this unique tissue.

Proposed Course

We will focus our studies for Fiscal Year 1994 on the following: (1) continuing the investigation of metal-catalyzed oxidation of lens proteins, (2) detailed characterization of the interaction of metals with crystallins and the effects on solubility, (3) analysis of human lens proteins in cataracts and the normal lens, and (4) molecular biology and immunological characterization of the enzyme that protects against thiol-dependent oxidation reactions.

NEI Research Program

Cataract—Lens Development and Aging

Publications

Bettelheim FA, Reid MB, Garland D: Hydration of gamma crystallins. *Exp Eye Res*, in press.

Giannessi M, Del Corso A, Cappiello M, Vatarelli M, Marini I, Barsacchi D, Garland D, Camici M, Mura U: Thiol-dependent metal catalyzed oxidation of bovine lens aldose reductase: I. Studies on the modification process. *Arch Biochem Biophys* 300:423-429, 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00272-03 LMOD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Inherited Ocular Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	James Fielding Hejtmancik	M.D., Ph.D.	Medical Officer	LMOD, NEI
Others:	John Hope	Ph.D.	Senior Fellow	LMOD, NEI
	Radha Ayyagari	Ph.D.	Special Volunteer	LMOD, NEI
	Ling Lee	M.S.	Chemist	LMOD, NEI
	Anthony Lloyd	M.D.	IRTA Fellow	LMOD, NEI
	T. Padma	Ph.D.	Visiting Scientist	LMOD, NEI

COOPERATING UNITS *(if any)*

Baylor College of Medicine (J. Towbin, B. Perryman, T. Ashizawa, P. Overbeek); Univ. of Iowa (R. Smith); Univ. of Texas-Houston (S. Daiger); Ocular Genetics and Clinical Services Branch, NEI, NIH (M. Kaiser-Kupfer); Washington Univ. at St. Louis (M. Petrash, R. Hayes); Massachusetts Institute of Technology (G. Benedek, J. Pande); Osmania Univ., Hyderabad, India (J.S. Murty)

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.15	5.15	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The study of inherited visual diseases provides a means by which both normal and aberrant visual processes might be understood. In addition to directly elucidating the pathophysiology of the inherited disease under study, these studies can provide insights into the structure-function relationships of the molecular components of the visual system and their normal physiology. This laboratory is using a number of approaches to study inherited visual diseases affecting the lens and retina.

Lens crystallins comprise over 90% of the soluble protein of the lens and are heavily modified in most cataracts. The effects which specific modifications of β- and γ-crystallin structure produce on crystallin functions, such as stability and formation of macromolecular aggregates, are being studied in tissue culture cells transformed with normal and modified βA3/A1-crystallin genes. Regions of the β-crystallin molecule of special interest include the amino terminal arm and the Greek key motifs of the core domains. The effects which these modifications have on lens transparency also are being studied in a transgenic mouse system in which a modified βA3/A1 gene is driven by an αA-crystallin promoter.

A second approach to understanding inherited visual diseases uses principles of positional cloning to identify genes important in human inherited diseases. Human diseases currently undergoing linkage analysis, gene isolation, or characterization of mutations include Usher syndrome, myotonic dystrophy, Duchenne muscular dystrophy, Long QT syndrome, cataracts, and a variety of X-linked syndromes. We currently are collecting families with autosomal recessive retinitis pigmentosa in preparation for study of this important group of diseases.

Project Description

Objectives

The long-range objectives of this project include increasing the understanding of inherited visual diseases, with the eventual aims of increasing the diagnostic ability for these diseases and providing a foundation for developing rational therapies based on a thorough knowledge of their molecular pathophysiology. These long-range objectives will be approached by pursuing the specific aims of identifying genes involved in inherited visual diseases and elucidating the mechanisms by which mutations in these genes cause disease.

Methods

Conventional cloning technology is utilized in preparing sequences for gene expression studies. These include ligation with T4 DNA ligase, screening by NaOH miniprep methodology, and ^{32}P-labeled DNA probes, as well as allele-specific oligonucleotide hybridization to screen for specific single-base settings. Sequence changes are introduced by site-specific mutagenesis via standard methodology. Gene expression in Chinese hamster ovary cells (RJK 88) and in insect cells (SF9) is enabled by the baculovirus expression system. Protein expression is monitored by standard two-dimensional gel electrophoresis followed by immunoblotting. Association behavior is assessed by elution volume on sieve FPLC.

Crystallin and other cDNAs and genomic fragments are isolated by library screening with cloned genes or oligonucleotides using routine methods. Sequencing is performed by cycling or using automated fluorescent technology (ABI).

Until recently, linkage analysis has involved conventional Southern blotting. Cell lines from NIH Eye Clinic patients and their family members are immortalized by Epstein Barr virus transformation. DNA is isolated by standard methodology and digested by restriction endonucleases. After agarose gel electrophoresis and Southern transfer, the resulting blot is probed with isolated DNA fragments labeled with ^{32}P by oligonucleotide labeling. Recently, short tandem repeat (microsatellite) markers have been analyzed by polymerase chain reaction performed in the presence of labeled oligonucleotides and analyzed on sequencing gels. Linkage data recorded on computerized spreadsheets are subject to both two-point and multipoint analysis with the LINKAGE program package.

Major Findings

1. The β-crystallins, their structure, and the mechanisms by which heterogeneity arises among this family of proteins are being investigated. The βA3-crystallin is identical to βA1 except for an additional 17-amino-acid N-terminal extension. The same gene is believed to encode and express both polypeptides. The βA3/A1 coding sequences were ligated behind the RSV promoter, and RJK 88 fibroblast cells were stably transfected with this construction. In addition, the βA3/A1 coding sequences were inserted into the Bluebac expression vector (Stratagene) and expressed in SF9 cells. A single 26-kD protein, the predicted size of βA1-crystallin, was detected on Western blots of soluble extracts of stable clones using antibodies raised to crystallin peptides. However, the RJK 88 cells, transformed with the same cDNA except with codons gln7 and leu10 mutated *in vitro* to stop codons, express only a 24-kD protein, the predicted size of the βA1-crystallin. Thus, it appears that the upstream (βA3) start codon is preferentially used in cell lines, although the downstream (βA1) start codon can be used.

In SF9 cells, a protein with the same amino terminal sequence as the βA1-crystallin is produced when the baculovirus-infected cells are grown past their prime. This is temporally correlated with the disappearance of the βA3-crystallin band, suggesting that the smaller band is created by processing or degradation of the larger in this system. In addition, clones for the mouse βA2-, βB1-, βB2-, and βB3-crystallin have been isolated and sequenced in preparation for characterization of their roles in β-crystallin aggregation.

2. We have constructed an additional crystallin in which the amino-terminal arm was deleted and replaced by a glycine residue, an extension identical to that found in γ2-crystallin. This new crystallin has been expressed in RJK 88 and SF9 cells (Bluebac vector) and has an appropriate migration on Laemmli gels, CD-spectrum, and amino acid sequence. The activity of this β-crystallin in association with the typical 200- to 250-kD aggregates has been tested by FPLC on superdex 75 and superose columns. The normal βA3 polypeptide readily associates into

homodimers, whereas the truncated βA3 associates minimally if at all. SF9 cells expressing the recombinant crystallins were grown in ^{35}S-containing medium, then purified and re-associated with an excess of lens extract containing normal crystallins (unlabeled) using limited urea denaturation followed by dialysis. Aggregation to form β-crystallin was assessed by FPLC on sizing columns. The recombinant full-length β-crystallin peptide aggregates into both dimers and tetramers, with the dimer peak migrating slightly before the β-light peak; however, the truncated βA3-crystallin migrates slightly behind the β-light peak and does not form obvious tetramers. These data strongly suggest that the amino-terminal arm of β-crystallins assists in the incorporation of β-crystallins into higher order aggregates.

3. We have constructed a βA3-crystallin in which the entire connecting peptide from the first to the second domain has been replaced with the corresponding sequence from γ2-crystallin. This construction should test the hypothesis that the connecting peptide, which crystallographic data show is extended in the β-crystallins and curved back on itself in the γ-crystallins, is responsible in this fashion for the β-crystallins' tendency to dimerize. The β-crystallin with the modified connecting peptide was subjected to the same tests of aggregation described in Section 2 above; it behaved essentially as the normal (unmodified) βA3-crystallin. The secondary structure of the modified β-crystallin currently is being confirmed with CD analysis.

4. Studies of phase transition properties of the γ-crystallin gene family have begun in collaboration with Drs. Mark Petrash (Washington University, St. Louis) and George Benedek (MIT, Boston). The bovine γB-crystallin has been modified at two of the four residues proposed to be critical for phase transition behavior. Phase transition analysis of the expressed unmodified γ2-crystallin has begun at MIT.

5. We also studied human genetic diseases that involve the eye. In addition to elucidating the pathogenesis of visual symptoms in inherited diseases, our efforts have provided reagents and information applicable to genomic analysis in general. Genetic markers in the myotonic dystrophy region have been used to confirm the diagnostic usefulness of bilateral lens opacities in the diagnosis of myotonic dystrophy; the data were confirmed by examining the trinucleotide repeat shown to be expanded in persons affected by myotonic dystrophy. The phenomenon of anticipation, long controversial in myotonic dystrophy, was shown to occur with statistical significance in the families enrolled in our study. In addition, earlier age of onset through anticipation was correlated with expansion of the trinucleotide repeat, although the correlation was not perfect. We have isolated a cDNA clone corresponding to the dystrophin gene product from a mouse lens library and are characterizing it.

6. Ophthalmologic diseases in humans have been studied by linkage analysis of RFLP markers. Diseases we have mapped within the past year include Long QT syndrome, X-linked agammaglobulinemia, and Usher's syndrome type 1. In addition, clinical and genetic heterogeneity of Usher's syndrome within the Acadian population of Louisiana has been explored in detail. Genetic analysis confirms the clinical impression that both type I and II of Usher's syndrome are found in the Acadian population, even within a single extended pedigree. The heterogeneity analysis described above implies this is due to segregation of two different, unlinked genes within this population.

Two genes causing Usher's syndrome type I have been mapped. In Acadians, the genetic locus is on chromosome 11p, while in the British families in our study, the gene is on chromosome 11q. When subjected to the most stringent heterogeneity analyses (both the HOMOG2 program and M test), these findings are significant at $p < 0.01$. This surprising finding implies that multiple genes can cause the rather specific clinical findings in Usher's syndrome. In detailed study of the Usher's syndrome gene on chromosome 11p, we have used fine linkage mapping and haplotype analysis to localize it to a 6-cM interval between the markers D11S861 and D11S928.

Several large families with autosomal dominant and recessive cataracts have been ascertained, and samples have been collected. Genotyping of microsatellite markers has begun for four of these families and will initially be concentrated in regions around candidate genes.

Significance to Biomedical Research and the Program of the Institute

Elucidation of the genetic defects causing visual disability will have implications far beyond the patient population suffering from the specific syndromes under study. Inherited diseases provide a means by which the molecular pathophysiology of

the visual system may be understood. This knowledge can then be applied to a broad spectrum of diseases. This rationale also applies to the study of inherited diseases of which visual defects are only a small part. Thus, while our studies of myotonic dystrophy already have resulted in improved diagnostic abilities, the mechanism by which cataracts occur in this disease will provide insight into cataractogenesis in other hereditary syndromes as well as in age-related and nonspecific cataracts.

Proposed Course

1. We will continue studies on the structure-function relationships of lens crystallins, concentrating on how modifications of the terminal arms and possibly the interconnecting peptide between the two domains affect aggregation of β-crystallins. We also will continue to explore the effects that modifications of the Greek key motifs have on crystallin stability and, when applicable, lens transparency. In addition, we will explore the effects of modifications of γ-crystallin sequences on the protein phase transitions and its relationship to cold cataract.

2. Sample collection and linkage analysis of a variety of human diseases will continue. The main emphasis will be on inherited visual diseases, especially Usher's syndrome type II. We are initiating a linkage study of autosomal dominant cataracts in families ascertained in collaboration with Dr. Muriel Kaiser (Ophthalmic Genetics and Clinical Services Branch) and of autosomal recessive cataracts ascertained in collaboration with Dr. J.S. Murty (Osmania University, India). This study will be coordinated with a new project to categorize and map expressed sequences of the human lens and the ongoing mechanistic studies on lens crystallins described above. Together these projects should provide a coordinated effort to elucidate the mechanisms of cataractogenesis in the human lens.

NEI Research Program

Cataract—Molecular Genetics

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00237-08 LMOD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of the Lens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Paul Russell	Ph.D.	Research Chemist	LMOD, NEI
Others:	Carolyn Chambers	Ph.D.	Senior Staff Fellow	LMOD, NEI
	Geoffrey Kidd	Ph.D.	Senior Staff Fellow	LMOD, NEI
	Santa Tumminia	Ph.D.	Senior Staff Fellow	LMOD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are continuing to develop an *in vitro* model to check anticataract agents. The organ culture system utilizes lenses from rats and monkeys. We have developed a method to screen the lenses to determine which might have been damaged in dissection. This is the first method to adequately predict the integrity of the lens at a very early stage in culture; lens clarity previously had been taken as a measure of integrity. Clarity at a very early stage in the culture process has not always been correct. Many lenses can maintain clarity but are not able to transport ions and amino acids normally. By analyzing the culture medium, we also have been able to show that the main protein in the organ culture medium is albumin, most probably as a result of residual protein sticking to the lens during dissection. The albumin can be taken out of the medium, and with a viable lens, very little protein will leak out of the lens. Curiously, when the lens is challenged with hydrogen peroxide as a model for oxidative stress, the βB-2 crystallin is one of the principal proteins lost by the lens. Additional studies of the organ-cultured lenses have begun analysis of the types of messenger RNA expressed in the lens under stress. We are using monkey lenses to determine the sequences of these stress-related messages.

Work on the lens epithelium has continued to study two specific questions: One concerns the protective mechanisms present in the lens epithelium to prevent damage from oxidative stress. Work with the lens epithelium cell lines has shown that the major oxidoreductase activated in an oxidative stress system appears to be D-T diaphorase. ζ-crystallin, also an oxidoreductase, is responsive to the oxidative stress and increases in the lens cells. The increase in the ζ-crystallin cannot by itself account for the large increase in oxidoreductase found in the stressed cells. The second question concerns cellular differentiation into fiber cells. The region of the lens where this process occurs tends to be the one most highly altered under stress conditions. We have separated certain steps in the differentiation process and will be better able to explore the stages at which cataracts might develop in the equatorial region of the lens.

Work continues on the human βB-2 crystallin, which now has been successfully cloned and sequenced. The deduced sequence recently has been confirmed by another lab. Since this protein is developmentally regulated, investigation into the promoter activity of this gene is continuing.

Project Description

Objectives

The purposes of this project are (1) to understand the basic biological processes of the human lens and how they are altered in cataract formation, (2) to develop model systems with which to mimic these processes, and (3) to use these model systems to develop methods to test anticataract agents.

Methods

Among numerous biochemical and molecular biological methods used in this research are Northern, Southern, and Western blotting of mRNA, DNA, and proteins. In addition, various methods for quantitation of these components, such as slot-blotting, are done. The polymerase chain reaction is used, as is nucleic acid sequencing.

Major Findings

1. An organ culture model system to test anticataract agents has been standardized. Part of the model involves screening the proteins in the culture medium to determine lens integrity.

2. With lenses from young animals, glutathione is rapidly lost in the organ culture system; however, this might not be the case with an older animal.

3. One of the major proteins that leak from the lens under conditions of stress is βB2-crystallin.

4. The βB2-crystallin has been cloned and sequenced from human lens. It has about 90% similarity with the mouse βB2-crystallin that we had sequenced previously. Our deduced sequence for the human crystallin has been confirmed by another group.

5. ζ-crystallin, an oxidoreductase found in guinea pigs and camels, has been found in the mouse lens. Lens cells from transgenic animals also have ζ-crystallin.

6. Lens cells under oxidative stress react to the stress by increasing oxidoreductase activity. The activity that appears to be most responsible for the amelioration of the effects of oxidative radicals is D-T diaphorase, although ζ-crystallin also is activated under oxidative stress conditions.

7. In tissue cultures, lens cells form so called "lentoid bodies." We have shown that lentoid body formation is an early step in the maturation process of the lens cell. The lentoid body can form without the activation of certain lens-specific proteins.

Lentoid body formation is similar to the elongation process that occurs in the equator of the lens. In this equatorial area, many cataracts originate. Thus, understanding the steps in the differentiation procedure is necessary to understand cataract formation.

8. Some of the proteins present in the aqueous humor of the eye have been identified. The aqueous humor is the fluid that nourishes the lens *in vivo*. The eight proteins that have been confirmed to be in the aqueous of the monkey are albumin, transferrin, ceruloplasmin, plasminogen, fibrinogen, α-1 antitrypsin, HDL, and cystatin.

Significance to Biomedical Research and the Program of the Institute

The development of systems to study the lens is vital to understanding the mechanisms involved in cataract formation. The new methods and protocols that we have developed for organ-cultured lenses have enabled us to standardize this useful technique for the study of cataract development. Working under definable, reproducible conditions is an advantage in formulating model systems to study conditions that lead to loss of cell function. These studies will enable us to devise systems to study anticataract agents.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00252-05 LMOD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cataract in the Philly Mouse Strain

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others:	Carolyn Chambers	Ph.D.	Senior Staff Fellow	LMOD, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00105-14 LMOD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure and Composition of Lens Crystallins with Respect to Cataractogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others:	Vasantha Rao	Ph.D.	Visiting Associate	LMOD, NEI
	Pedro Gonzalez	Ph.D.	Visiting Fellow	LMOD, NEI
	Chuan Qin	M.D.	Visiting Fellow	LMOD, NEI

COOPERATING UNITS *(if any)*

Jules Stein Eye Institute, UCLA (J. Horowitz, B. Bateman); Laboratory of Molecular and Developmental Biology, NEI (G. Wistow, D. Lee); National Cancer Institute (M. Krishna); Centre for Cellular and Molecular Biology, Hyderabad, India (D. Balasubramanian; M. Rao)

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Cataracts

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project, directed toward elucidation of the molecular mechanisms responsible for cataractogenesis, places special emphasis on the role of the structure and function of the lens crystallins. Until recently, the crystallins were thought to be simply structural elements of the lens protein matrix, without any specific quantifiable biological function. Two recent discoveries have provided new insights and approaches to the physiological roles of the crystallin: (1) the crystallins either are functionally active enzymes or are at least related to proteins with specific biological activities and (2) α-crystallin is a molecular chaperone that can prevent the aggregation of denaturing proteins.

Our group is studying the chaperone function of α-crystallin, with the goal of establishing its significance in the intact lens. Using the isolated crystallin, we have shown that it forms stable complexes with target proteins, specifically interacting with denaturing proteins at the earliest stage of denaturation. The specificity of this interaction has been demonstrated by the fact that in some instances it is strongly dependent on the availability of obligate cofactors of the target proteins. Studies on "enzyme/crystallins" focus on ζ-crystallin, a major protein in the lenses of certain mammals (e.g., guinea pigs, camelids). In guinea pigs a mutation in the ζ-crystallin gene causes hereditary nuclear cataracts, and our goal is to understand how the mutation affects the lenticular function(s) of the protein, leading to cataract. The ζ-crystallin system also is being used to investigate the mechanisms of lens-specific expression of crystallin genes.

Lens organ culture is being used as both a means of testing potential anticataract agents and a system for analyzing the responses of intact lenses to various cataractogenic stresses. The changes in gene expression induced in primate lenses by stress are being studied to identify specific proteins and processes important in combating stress. This information will facilitate more rational design of strategies to accomplish our ultimate goal: the prevention or delay of cataractogenesis.

Project Description

Objectives

The primary objectives of this project are (1) to elucidate at the molecular level processes responsible for cataract development, (2) to investigate the structures and functions of the lens crystallins, and (3) to develop and use model systems for screening potential anticataract agents.

Methods

Conventional protein chemical techniques employed are chromatography, electrophoresis, and isoelectrofocusing. Immunological studies of lens proteins use specific antisera. Physicochemical analyses on the proteins are performed using high-pressure liquid chromatography, fluorescence, and circular dichroism techniques. In lens organ culture experiments involving rat or monkey lenses, we use active transport and membrane permeability parameters to monitor the effects of various stresses on the cultured lenses.

Techniques used in analysis of nucleic acids include RNA and DNA isolation, cDNA and gene cloning, DNA sequencing, various electrophoretic methods, and the polymerase chain reaction.

Major Findings

1. α-crystallin acts as a molecular chaperone, forming stable complexes with various other proteins undergoing denaturation and preventing their aggregation. Once fully denatured, the target proteins do not associate with α-crystallin. Thus, like other chaperone proteins, α-crystallin specifically recognizes and binds proteins only in the very early stage of denaturation.

2. Further evidence for the specificity of this reaction is provided by the finding that, with certain proteins, protection from aggregation by α-crystallin is dependent on the presence of cofactors. For example, ζ-crystallin/quinone reductase, an NADPH-requiring enzyme, is efficiently protected only in the presence of NADPH.

3. The ζ-crystallin cDNA from the lens of the llama has been sequenced and found to be highly similar to that of other mammals analyzed. The llama is a camelid and therefore of particular interest because, like guinea pigs, these animals have very high levels of lenticular ζ-crystallin.

4. Our analysis of the llama ζ-crystallin gene has revealed two promoters, one of which regulates normal low level expression in many tissues. This promoter exists in all ζ-crystallin genes examined. A second lens-specific promoter is found only in species in which the protein is also a major lens protein (e.g., guinea pig and llama). Interestingly, the promoter in the llama gene is unrelated to the lens-specific promoter previously characterized in the guinea pig, suggesting that ζ-crystallin/quinone reductase was recruited as a lens protein at least two different times during evolution.

5. The human ζ-crystallin gene has been localized to chromosome 1p22-p31[2], and six restriction fragment-length polymorphisms (RFLPs) have been identified within the gene.

6. Analysis of the sequences of all known lens crystallins reveals that, in general, they are not designed for high intracellular (metabolic) stability. Therefore, the extremely long half-lives of crystallins must result largely from the environment within the lens rather than from intrinsic properties of the proteins themselves.

7. The organ-cultured rat lens loses 40% of its glutathione (GSH) during the first 24 hours of culture and more than 60% by 72 hours. This loss occurs even in the absence of O_2 and, thus, is not the result of oxidative stress in the culture system. Interestingly, monkey lenses cultured for up to 48 hours showed no decrease in GSH.

8. Fluorescence spectra of intact human lenses over a wide age range demonstrate different amounts and numbers of fluorophors in lenses from the United States relative to lenses collected and analyzed in India. It is hoped that these analyses will give clues to the molecular mechanisms underlying the increased pigmentation and earlier onset of cataract in India.

Significance to Biomedical Research and the Program of the Institute

Cataract is a major public health problem worldwide. Better understanding of the biochemistry of the normal lens and of the molecular changes that occur during aging and cataract development are essential if this disease is to be controlled. Our studies are aimed primarily at elucidating the role of the lens crystallins, the primary structural elements of the normally transparent lens matrix, in the processes

leading to opacification. Such knowledge should contribute to the development of means of intervention that can prevent or delay the process of cataract development.

Proposed Course

We will continue to (1) work to establish viable model systems for testing anticataract agents and use these systems to assess the efficacy of various types of compounds, including antioxidants, (2) complete analysis of the molecular basis underlying the high lens-specific expression of an enzyme/crystallin (ζ-crystallin), (3) further investigate the chaperone-like function of α-crystallin and determine its physiological significance in the normal lens and in cataract, and (4) evaluate gene expression in lenses under stress to seek proteins critical in the response to stress.

NEI Research Program

Cataract—Pathogenetic Mechanisms

Publications

Gonzalez P, Rao PV, Zigler JS Jr: Molecular cloning and sequencing of zeta-crystallin/quinone reductase cDNA from human liver. *Biochem Biophys Res Commun* 191:902-907, 1993.

Jörnvall H, Persson B, Du Bois GC, Lavers GC, Chen JH, Gonzalez P, Rao PV, Zigler JS Jr: Zeta-crystallin versus other members of the alcohol dehydrogenase superfamily: Variability as a functional characteristic. *FEBS Lett* 322:240-244, 1993.

Lee DC, Gonzalez P, Rao V, Zigler JS Jr, Wistow GJ: Carbonyl-metabolizing enzymes and their relatives recruited as structural proteins in the eye lens. *Adv Exp Med Biol* 4:159-168, 1993.

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Tumminia SJ, Rao Pv, Zigler JS Jr, Russell P: Xenobiotic induction of quinone oxidoreductase activity in lens epithelial cells. *Biochim Biophys Acta*, 1203:251-253, 1993.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00149-20 LMOD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ultrastructure and Function of the Cells and Tissues of the Eye

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W. Gerald Robison, Jr.	Ph.D.	Head, Section on Pathophysiology	LMOD, NEI
Others:	Nora Laver	M.D.	Special Volunteer	LMOD, NEI
	Anne Groome	B.S.	Histology Technician	LMOD, NEI
	Joe Hackett	B.S.	Biologist	LMOD, NEI
	Evita Bynum	B.S.	Microbiologist	LMOD, NEI
	Joel Glover	B.S.	Biologist	LMOD, NEI

COOPERATING UNITS *(if any)*

Alcon Laboratories, Inc. (Billie M. York, Jr., Ph.D.)

LAB/BRANCH

Laboratory of Mechanisms of Ocular Diseases

SECTION

Section on Pathophysiology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.75	1.0	3.75

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Using the galactose-fed rat model for diabetic retinopathy, which was first developed in this laboratory, we designed intervention studies to test the possibility of delaying, halting, or reversing retinopathy soon after the earliest capillary lesions could be documented. Weanling male SD rats were divided into five groups, three of which received either normal lab chow or a 50% galactose diet with or without an aldose reductase inhibitor (ARI:ca. 11 mg/kg/day AL-3152) and two of which received 50% galactose for 6 months and then intervention, by either addition of inhibitor or removal of galactose. From each rat killed at 6, 18, and 24 months, one retina was prepared for obtaining electron micrographs of capillary transections and the other was used for whole mounts of isolated retinal vessels. We captured images of whole and transected capillaries and analyzed them using computer hardware and programs specially designed for 1,024 × 1,024 × 8-bit resolution. Based on several quantitative assessments, including basement membrane thickness, PAS stain intensity, acellularity, dilation, tortuosity, length, and microaneurysms, the retinopathy was graded on a scale of 1 to 10. At 6 months, when intervention began, untreated galactose-fed rats exhibited a 30%, statistically significant ($p < 0.01$) increase in capillary basement membrane thickness and grade-1 retinopathy overall. By 18 months, the same group had grade-7 retinopathy whereas rats receiving intervention with either AL-3152-enriched or galactose-free diets exhibited only grade-2 retinopathy, and rats fed control diet or galactose plus AL-3152 throughout 18 months showed none. At 24 months, untreated rats had grade-10 retinopathy, and both intervention groups had grade-8 retinopathy. Thus, intervention at 6 months delays but does not halt or reverse the progression of galactose-induced retinopathy.

We plan to attempt, by dietary manipulation, to produce rat models that develop the diabetic-like retinal angiopathies sooner. Also, using cell culture, we will investigate possible mechanisms of endothelial cell proliferation and subsequent pathologies.

Project Description

Objectives

This project is designed to use special diets *in vivo* and controlled media in cell cultures of ocular tissues to mimic the diabetic state in order to determine whether diabetic-like tissue changes can be prevented by aldose reductase inhibitors (ARIs).

Methods

Weanling male Sprague-Dawley rats were divided into five groups, three of which received either normal lab chow or a 50% galactose diet, with or without an ARI (ca. 11 mg/kg/day AL-3152), and two groups which received 50% galactose for 6 months and then intervention by either the addition of an inhibitor or the removal of galactose. Rats were killed at 6, 18, and 24 months. A new enzyme digestion procedure (elastase method) developed in this lab was used on the retina of one eye of each rat to remove all retinal tissues except the vessels. This provided a whole mount of the retinal vasculature and permitted the recognition of degenerated pericytes ("ghosts") and all of the more advanced angiopathies by light microscopy. The retina of the other eye of each pair was sectioned and examined by electron microscopy. Images of whole and transected capillaries were captured and analyzed by using computer hardware and programs specially designed for 1024 × 1024 × 8-bit resolution. Based on several quantitative assessments—including basement membrane thickness, PAS stain intensity, acellularity, dilation, tortuosity, length, and microaneurysms—the retinopathy was graded on a scale of 1 to 10. Tissue cultures of human, bovine, and canine retinal capillary pericytes and lens epithelial cells were used to investigate the mechanism(s) underlying the diabetic angiopathies.

Major Findings

Vascular whole mounts of capillaries of rats fed galactose for 24 months, prepared by our new enzyme digestion procedure, exhibited multiple retinal angiopathies identical to those typical of human background diabetic retinopathy. These angiopathies did not occur in the retinas of rats fed a galactose diet with an ARI. The presence of aldose reductase was demonstrated in cultured retinal pericytes (1) by immunohistochemistry, shown by the antibody against human placental aldose reductase; (2) by its activity, shown by measurements of xylitol production in cells grown in a medium supplemented with xylose; and (3) by the detection of messenger RNA for aldose reductase. There was a compromised proliferation rate in pericytes, compared with endothelial cells incubated in high (30 mM) sugar concentrations, suggesting toxicity of polyol at the cellular level. Aldose reductase appears to be involved in all the retinal complications of diabetes, from pericyte degeneration to microaneurysms.

Significance to Biomedical Research and the Program of the Institute

Diabetic retinopathy is mainly a disease of retinal capillaries. Recently potentially beneficial treatments and animal models have become available. However, demonstration of the earliest vessel lesions has relied on the 30-year-old trypsin digestion method for the isolation of retinal vessels. Until now, basic experimental studies and drug testing on diabetic retinopathy have been limited by the lack of reliable and convenient animal models. Now, besides the alloxan diabetic dog and the galactosemic dog, there is a galactosemic rat model. All this has been possible because aldose reductase is involved in diabetic retinopathy. Aldose reductase, which has been implicated in sugar cataracts, certain corneal healing defects, and peripheral neuropathy of diabetic and galactosemic animals, now appears to be involved in all lesions of background diabetic retinopathy. While the normal physiological role of this enzyme in most tissues remains unknown, under the conditions of high plasma sugar concentrations encountered in diabetes and galactosemia, aldose reductase converts these sugars to their respective sugar alcohols (polyols). These polyols are not readily metabolized, nor do they penetrate cell membranes easily. Thus, once formed at significant rates, they may accumulate to very high levels in cells, leading to hypertonicity, alteration of ion permeability, and eventual cell death, with consequent tissue changes such as cataract formation. Treatment of diabetic or galactosemic rats with potent ARIs, such as sorbinil or tolrestat, decreases the accumulation of polyols, which in turn appears to prevent the formation of cataracts in lenses, defective healing in scraped corneas, thickening of basement membranes in retinal capillaries, and decreased conduction velocity in nerves.

By using a novel vessel preparation method, we have shown for the first time that the rat can be a good model for human diabetic retinopathy and that demonstration of early lesions can be improved. Pericyte loss, endothelial cell proliferation, microaneurysms, shunts, occlusions, dilations, and all the other microangiopathies that we found in the galactose-fed rat are identical to the histopathologies that characterize human background diabetic retinopathy. Until now, the only other experimental animal model has been the diabetic or galactosemic dog. We have shown for the first time that diabetic-like retinopathy in galactosemic rats can be prevented with an ARI.

Proposed Course

The following studies are proposed for Fiscal Year 1993. We will extend the intervention studies to determine how late one can interrupt the disease process and still obtain beneficial results by treatment with various ARIs. We also will examine the early formation of intracellular vacuoles, cell transport systems, the mechanism of basement membrane synthesis, and the relationships of these changes to aldose reductase in isolated retinal cells grown under diabetic conditions. We will manipulate the rat diets to shorten the time when diabetic-like retinal angiopathies appear, thus improving the rat as a model for diabetic retinopathy.

NEI Research Program

Retinal Diseases—Diabetic Retinopathy

Publications

Laver NM, Robison WG Jr: Proliferative retinopathy stage in long-term galactose fed rats. *Invest Ophthalmol Vis Sci* 34(4)(suppl):713, 1993.

Laver N, Robison WG Jr, Calvin HI, Fu S-CJ: Early epithelial lesions in cataracts of GSH-depleted mouse pups. *Exp Eye Res* 57:493-498, 1993.

Laver NM, Robison WG Jr, Hansen BC: Demonstration of retinal histopathologies in spontaneously diabetic monkeys. *Am J Clin Pathol* 99(3): 349, 1993.

Laver NM, Robison WG Jr, Pfeffer BA: Novel procedures for isolating intact retinal vascular beds from diabetic humans and animal models. *Invest Ophthalmol Vis Sci* 34:2097-2104, 1993.

Matthews GP, Laver N, Robison WG Jr: Electrophysiological and histological evaluation of inner retina in galactosemic rats. *Invest Ophthalmol Vis Sci* 34(4)(suppl):720, 1993.

Robison WG Jr, Laver N: Ocular lesions in animal models of human diabetes, in Shafrir E (ed): *Frontiers in Diabetes Research, Lessons from Animal Diabetes IV.* London, Smith-Gordon and Company Limited, 1993, pp 145-163.

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Laboratory of Molecular and Developmental Biology

Report of the Chief, Laboratory of Molecular and Developmental Biology

Joram Piatigorsky, Ph.D.

In its 12th year, the Laboratory of Molecular and Developmental Biology (LMDB) has been expanded by two sections—the Section on Regulation of Gene Expression, headed by Dr. Ana B. Chepelinsky, and the Section on Transgenic Animals and Genomic Manipulation, headed by Dr. Eric Wawrousek. Dr. Chepelinsky, a valued member of the LMDB since its beginning, has augmented her research area, the crystallins, to include membrane proteins and their genes, as well as the effects of growth factors on eye development. Dr. Wawrousek, a former postdoctoral fellow at the LMDB, has returned to the National Eye Institute (NEI) after a brief sojourn in industry. His Section wears two hats. The first provides a service for the NEI—the creation of transgenic mice; the second performs research involving site-specific gene recombination. The addition of these two sections has increased the expertise of the LMDB and extended our usefulness to the NEI. The other sections of the LMDB include the Section on Molecular Genetics, headed by Dr. Joram Piatigorsky; the Section on Cellular Differentiation, headed by Dr. Peggy S. Zelenka; and the Section on Molecular Structure and Function, headed by Dr. Graeme J. Wistow.

Not all developments are happy ones. Sadly, Ms. Dawn Chicchirichi, the LMDB secretary since the Laboratory's beginning, retired due to illness. Ms. Chicchirichi gave 11 years of devoted and excellent assistance to the LMDB and will be greatly missed. She has been replaced by Ms. Linda Willett. Ms. Willett already has become an invaluable member of the LMDB, and we are extremely lucky to have her with us. I also take this opportunity to thank the many NEI staff members who gave us great support and help during the difficult months of Ms. Chicchirichi's illness so that we could keep functioning during the transition period.

The LMDB's primary goal is to perform basic research on the molecular biology of the eye. Although particular attention is directed to the lens, the cornea and retina have not escaped our efforts; research projects also have focused on the role of growth factors on eye development. In addition, because lens crystallins are multifunctional proteins that are expressed outside the lens and eye, the scope of our research has increased during the last few years to include new areas of metabolism and gene expression in various tissues. Moreover, many of the transcription factors involved in the expression of crystallin genes are present in many tissues and are used to control numerous biological processes. Consequently, our studies on eye genes have implications for many areas of molecular, cellular, and evolutionary biology. This is reflected in the plethora of general journals in which we publish our scientific discoveries and the fact that we often attend meetings that focus on broad issues of genetics, development, evolution, and molecular biology. Thus, the original twin purposes of using the visual system as a model for the structure, expression, and evolution of genes and incorporating general principles of molecular biology to understanding the visual system continue as the core of our thinking.

There have been many individual research accomplishments by LMDB staff this year. These accomplishments are detailed in the specific annual reports. In general, much attention has been given to the identification of regulatory elements required for expression of genes in the eye and other tissues. Many of these regulatory elements are commonly found in genes; however, each has its own special properties. The diversity of elements used for expression of eye genes is impressive, ensuring that we will be busy for many years sorting them all out. To complicate things even more, we have shown that the regulatory elements may be functionally redundant, i.e., removing one does not necessarily eliminate the expression of the gene. There are also many different nuclear proteins that bind to the DNA regulatory elements, and this year we have cloned a number of them. One of our biggest challenges is to determine which binding proteins are actually involved in regulating the genes in the animal.

Our studies on the expression of proto-oncogenes and cyclins have linked the normal process of cellular differentiation in the lens with the cell cycle and growth control, providing another example of the broad relevance of our research. In addition, the use of crystallin promoters for directing various growth factors to the lens have extended cellular studies to consideration of growth of the entire eye. These genetic engineering experiments have opened the opportunity to develop animal models for autoimmune diseases of the eye, fostering communication between the LMDB and the NEI Laboratory of Immunology. The addition of the transgenic facility has had a major impact in increasing the dialog between the LMDB and other NEI laboratories. We look forward to additional cross-fertilization of ideas in the years to come.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00238-08 LMDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Proto-Oncogene Expression During Lens Differentiation and Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Peggy S. Zelenka	Ph.D.	Head, Section on Cellular Differentiation	LMDB, NEI
Others:	Jo Ann Rinaudo	Ph.D.	Staff Fellow	LMDB, NEI
	Chun Yun Gao	M.D., Ph.D.	Staff Fellow	LMDB, NEI
	Emmanuel Vacchiano	Ph.D.	Staff Fellow	LMDB, NEI
	Anuradha Rampalli	Ph.D.	Visiting Fellow	LMDB, NEI
	Jaspreet Arora	Ph.D.	Visiting Fellow	LMDB, NEI
	Graeme Wistow	Ph.D.	Head, Section on Molecular Structure and Function	LMDB, NEI

COOPERATING UNITS *(if any)*

Department of Surgery, New Jersey Medical and Dental College (Thomas Lysz, Ph.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Cellular Differentiation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.2	5.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project to investigate the expression of proto-oncogenes and other cell cycle regulatory genes in the embryonic chicken lens seeks to determine their relationship to cell growth, quiescence, and differentiation. The normal developmental profiles of five nuclear proto-oncogene mRNAs (c-myc, N-myc, c-fos, c-jun, and p53) and the cell cycle regulatory protein, cyclin B, have been completed, and the profile of retinoblastoma (Rb) expression is in progress. The finding that cyclin B is present in lens-fiber cells suggests that lens-fiber cell differentiation may represent an aberrant form of the cell cycle. In addition to cyclin B, a number of other proteins normally associated with proliferating cells are expressed in postmitotic, differentiating lens cells. These include cyclin A, c-myc, c-fos, c-jun, and p53. Moreover, preliminary studies using explanted embryonic chicken lens epithelia indicate that the order of expression of these genes during differentiation is the same as during proliferation, further strengthening the link between differentiation and the cell cycle. The functional role of each of these genes during lens differentiation now is being explored through the use of retroviral vectors, transfection of exogenous DNA, and the production of transgenic mice. In addition, regulatory mechanisms governing the changes in proto-oncogene expression that accompany differentiation are being explored.

Project Description

Additional Personnel

Milton Berger	B.A.	Special Volunteer, LMDB, NEI
Tania Tolstoshev		H.S. Student, Special Volunteer, LMDB, NEI
Graeme Wistow	Ph.D.	Head, Section on Molecular Structure and Function, LMDB, NEI

Objectives

In this project we seek to determine whether the expression of specific proto-oncogenes is altered during lens cell differentiation and, if so, to determine the mechanism of gene regulation and the function of the corresponding proto-oncogene products in the developing lens. The objective is to develop a greater understanding of the mechanisms underlying lens cell growth and differentiation.

Methods

Techniques of molecular biology are used in conjunction with traditional cell biology techniques. Conventional methods are employed for protein and nucleic acid analysis, including polyacrylamide gel electrophoresis, RNA and DNA isolation, polymerase chain reaction (PCR), reverse transcription PCR (RT/PCR), nucleic acid hybridization, *in vitro* transfection, *in situ* hybridization, immunocytochemistry, and immunoblotting. DNA/protein interactions are studied using DNAse I footprinting, electrophoretic mobility shift assays, and ultraviolet (UV) cross-linking.

Studies employ lens epithelia and lens fibers of embryonic chickens, explants of embryonic chicken lens epithelia, primary cultures of embryonic chicken lens epithelial cells, and other avian and mammalian cell lines. In addition, transgenic mice are produced to test the function of proto-oncogenes and cell cycle regulatory proteins in the lens *in vivo*.

Major Findings

In the past year major progress has been made on studies of the cell cycle regulatory protein, cyclin B. Expression of this protein is known to be cell-cycle dependent in proliferating cells, appearing in the S and G2 phases of the cell cycle. Work done by Dr. Chun Gao has demonstrated that cyclin B is expressed in differentiating lens fiber cells. The presence of cyclin B mRNA was shown by RT/PCR, followed by sequencing of the PCR product. Immunoblotting with an antibody specific for cyclin B following two-dimensional gel electrophoresis confirmed that the protein is also present in lens fiber cells.

In situ hybridization of sections of 14-day embryonic lenses with riboprobes for cyclin B mRNA showed that the mRNA is abundant in the differentiating cells at the lens equator and in the nucleated fiber cells, but it cannot be detected in the enucleated fiber cells. Cyclin B from lens fiber cells was affinity-purified by chromatography on $p13^{suc1}$ Sepharose. Because the p13 protein binds to $p34^{cdc2}$ kinase, purification of cyclin B by this process provides evidence that it is complexed with the $p34^{cdc2}$ protein. The kinase activity of this complex was demonstrated by using histone H1 as a substrate. Kinase activity could be increased about twofold by phosphatase treatment.

These results indicate that cyclin B, a protein normally expressed in the G2 phase of the cell cycle, is present in differentiating lens fiber cells. Since the cyclin B/$p34^{cdc2}$ complex is known to be responsible for chromosomal condensation and nuclear envelope breakdown in mitotic cells, finding this complex in lens fibers and in the enzymatically active, dephosphorylated form provides evidence that this same biochemical mechanism may be responsible for chromosomal condensation and nuclear envelope breakdown during fiber cell differentiation.

Because cyclin B normally is expressed only in the G2 phase of the cell cycle, its presence in differentiating lens fiber cells suggested that the differentiation process itself may be an aberrant form of the cell cycle. To test this possibility, Dr. Anuradha Rampalli has initiated experiments to examine the order of expression of a number of cell-cycle markers in differentiating explants of 6-day-old embryonic chick lens epithelia. Preliminary results show a strong, early induction of c-fos, c-jun, c-myc, and N-myc, followed after a lag of 5-7 hours by induction of p53 and after a lag of 18-24 hours by induction of the heat shock protein HSP70. Since c-fos, c-jun, and c-myc are normally expressed in early G1, p53 in late G1, and HSP70 during the S and G2 phases in proliferating cells, the order of induction seen

during differentiation parallels that of the normal cell cycle. Additional S-phase markers under investigation include PCNA (the δ subunit of DNA polymerase) and thymidine kinase. Cyclins A and B will be examined as markers for the G2 phase. The induction of N-myc is not typical of proliferating cells and, thus, marks a significant difference between the two processes.

The tumor supressor genes Rb and p53 seem to play key roles in preventing G1 cells from entering the S phase, making their role in lens differentiation particularly interesting. Studies by other investigators have shown that inactivation of these gene products by SV40 T antigen prevents terminal differentiation of lens fiber cells. Dr. Rampalli has completed a developmental study of p53 expression in the embryonic chick lens, and a companion study of Rb expression is in progress. Her results clearly show that p53 mRNA and protein are expressed in differentiating cells at the lens equator and in the newly formed fiber cells, consistent with the data from differentiating explants and with the idea that differentiation and cell-cycle progression share important features.

A major focus of this project continues to be the biological function of the proto-oncogenes expressed in the lens. Preliminary evidence, reported last year, that c-myc regulates expression of the τ-crystallin/α-enolase gene has now been extended to demonstrate that the c-myc protein itself is involved in binding to the τ-crystallin promoter. Interestingly, a τ-crystallin/chloramphenicol acetyltransferase (CAT) construct with a mutation in the potential c-myc binding site was shown to be expressed at somewhat higher levels in cultured lens cells than was the wild-type construction, although cotransfection of a c-myc expression vector was no longer required to stimulate this expression. This observation raises the possibility that c-myc and a negative regulatory protein may compete for binding to the same or overlapping sites. The possibility that N-myc may be such a negative factor is under investigation.

The function of c-jun in the embryonic chicken lens has been explored using wild-type and mutated cDNAs for chicken c-jun cloned into the avian retroviral vector RCAS. Use of this vector permits transfer of the c-jun constructs to cultured cells with efficiencies approaching 100%, making it possible to test for the effects of c-jun on DNA synthesis, differentiation, and expression of endogenous genes. Results obtained by Drs. Jo Ann Rinaudo and Emmanuel Vacchiano indicate that a negative dominant mutation of c-jun increases the levels of endogenous αA-crystallin mRNA twofold above the already high levels present in the control cells. The levels of βA3/A1 mRNA were not affected in the same cells, suggesting that multiple, independent pathways may operate in differentiating lens cells, only some of which are affected by c-jun.

Overexpression of wild-type c-jun in chicken lens epithelial cells by means of the RCAS vector greatly enhances cell proliferation and may immortalize the cells. Cells infected with a retrovirus bearing the c-jun cDNA have now undergone nine passages, with no apparent decrease in proliferative capacity. Furthermore, the cells seem to have retained their ability to differentiate to lens fibers; lentoid bodies form in the cultures when they are permitted to become confluent. If these cells are immortalized, they will be extremely useful for future studies of gene expression and differentiation.

Noting the similarities between lens fiber cell differentiation and apoptosis, Dr. Vacchiano has employed the c-jun retroviral vector to investigate the role of c-jun in proliferation, differentiation, and apoptosis in chicken embryo lens epithelial cells. His results indicate that c-jun overexpression stimulates proliferation in the presence of serum, but it does not prevent differentiation once confluence is attained. In the absence of adequate levels of serum or growth factors, however, overexpression of c-jun increases the rate at which the cells enter apoptosis.

Dr. Vacchiano also is investigating the possible role of bcl-2 expression in regulating lens cell growth and differentiation. This proto-oncogene has been shown in other cell types to block apoptosis induced by overexpression of c-myc or p53. Using RT/PCR, he has demonstrated that bcl-2 is expressed in the embryonic chicken lens. He is now preparing constructs that will permit overexpression of this gene in both cultured chicken lens epithelial cells and transgenic mice to determine its effect on apoptosis and differentiation of lens cells. Inhibition of differentiation by bcl-2 would indicate an important biochemical link between fiber cell formation and apoptosis.

Our ongoing collaboration with Dr. Thomas Lysz (University of Medicine and Dentistry of New Jersey) has now demonstrated that endogenous production of 12-hydroxyeicosatetraenoic acid (12-

HETE), a lipoxygenase pathway metabolite of arachidonic acid, is a required step in DNA synthesis stimulated by epithelial growth factor in the neonatal rat lens. Dr. Jaspreet Arora has used RT/PCR to demonstrate that 12-HETE synthesis is required for expression of two proto-oncogenes, c-fos and c-myc, whereas expression of c-jun seems to be independent of this pathway. Because inhibition of either c-fos or c-myc is sufficient to cause cell-cycle arrest, these findings indicate that 12-HETE production is a key control point in the lens epithelial cell cycle.

Significance to Biomedical Research and the Program of the Institute

The proto-oncogenes are normal cellular homologs of retroviral oncogenes. Since retroviral transformation disrupts cell growth and differentiation, it is likely that the proto-oncogenes are involved in the normal regulation of these processes. A study of proto-oncogene expression during lens cell differentiation may elucidate basic regulatory processes underlying lens cell growth and differentiation. Many types of cataract are associated with abnormal lens epithelial cell growth and inhibition of lens fiber cell differentiation. In addition, a number of other eye diseases involve loss of normal controls on cell proliferation. An understanding of the basic controls of cell growth and differentiation would further our understanding of these disease states.

Proposed Course

The following studies are in progress or are proposed for Fiscal Year 1994:

1. We will test the hypothesis that the cyclin B/$p34^{cdc2}$ kinase is responsible for nuclear loss in differentiating lens cells by producing transgenic mice that express the $Wee1^{+}$ kinase in lens fiber cells. This kinase inactivates the cyclin B/$p34^{cdc2}$ kinase and would be expected to delay or prevent nuclear loss if cyclin B/$p34^{cdc2}$ is required.

2. Due to the unexpected finding of the cyclin B/$p34^{cdc2}$ kinase in differentiating lens fibers and the noted similarities between lens differentiation and apoptosis, we will examine whether cyclin B is expressed in apoptotic cells. We will test a variety of apoptotic cells of divergent origins for the presence of cyclin B and cyclin B/$p34^{cdc2}$ kinase activity.

3. We will continue study of the time course of the expression of cell-cycle-dependent genes in differentiating explants of lens epithelia, with the addition of S and G2 phase markers to determine the extent to which differentiation resembles cell-cycle progression.

4. We will examine further the effect of c-myc on transcription of the τ-crystallin/α-enolase gene by transfection studies in cells that do not express N-myc. We also will examine the effect on the endogenous duck gene by transfection into duck fibroblasts and lens epithelial cells.

5. We will examine the effect of the N-myc proto-oncogene on transcription directed by the τ-crystallin/α-enolase gene using the techniques previously used to study the role of c-myc.

6. We will extend the collaborative effort with Dr. Lysz to other growth factors, as well as examine the mechanism by which 12-HETE affects expression of c-fos and c-myc. We will experiment to determine whether human lenses possess the 12-lipoxygenase pathway.

7. We will explore the possible role of post-translational modifications of Rb and p53 proteins in lens cell differentiation.

NEI Research Program

Cataract—The Normal Lens

Publications

Dash A, Chung S, Zelenka PS: Expression of HSP70 mRNA in the embryonic chicken lens: Association with differentiation. *Exp Eye Res*, in press.

Piatigorsky J, Zelenka PS: Transcriptional regulation of crystallin genes: cis elements, trans-factors, and signal transduction systems in the lens. *Adv Develop Biochem* 1:211-256, 1992.

Wistow GJ, Shaughnessy MP, Lee DC, Hodin J, Zelenka PS: A macrophage migration inhibitory factor is expressed in the differentiating cells of the eye lens. *Proc Natl Acad Sci USA* 90:1272-1275, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00126-12 LMDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Crystallin Genes: Structure, Organization, Expression, and Evolution

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Joram Piatigorsky	Ph.D.	Chief	LMDB, NEI
Others:	James B. Brady	Ph.D.	IRTA	LMDB, NEI
	Sambath Chung	B.A.	Technician	LMDB, NEI
	Ales Cvekl	Ph.D.	Visiting Fellow	LMDB, NEI
	Melinda Duncan	Ph.D.	IRTA	LMDB, NEI
	Peter Frederikse	Ph.D.	Senior Staff Fellow	LMDB, NEI
	Rashmi Gopal-Srivastava	Ph.D.	Staff Fellow	LMDB, NEI
	John Haynes	Ph.D.	IRTA	LMDB, NEI
	John G. Ilagan		Howard Hughes Medical Institute/ NIH Fellow	LMDB, NEI

(Additional personnel listed under Program Description.)

COOPERATING UNITS *(if any)*

Jules Stein Eye Institute, UCLA (J. Horwitz, Ph.D.); National Institute of Child Health and Human Development, NIH (K. Becker, Ph.D.; K. Ozato, Ph.D.); University of Southern California (V.M. Weis, Ph.D.; M. McFall-Ngai, Ph.D.); Medical College of Virginia (D.M. Stover, Ph.D.; Z.E. Zehner, Ph.D.); N.K. Koltzov Developmental Biology Institute, Russian Academy of Sciences, Moscow (R.D. Zinovieva, Ph.D.); Uniformed Services University of the Health Sciences (S. Bassnet, Ph.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Molecular Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
14.0	10.8	3.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structure, expression, and evolution of crystallin genes of vertebrates and invertebrates are being studied. The four functional promoter elements described in the αA-crystallin gene of the mouse (DE1, αA-CRYBP1, PE1, and PE2) and five in that of the chicken (DE3, DE2A, DE2B, DE1A, and DE1B) are surprisingly different, considering the gene is orthologous in these species with the same high-level expression in the lens. We have cloned putative *trans*-acting factors which bind to the mouse αA-CRYBP1 and chicken DE2A sites. The αA-CRYBP1 gene has been cloned and characterized; cDNA analyses indicate that it produces alternatively spliced mRNAs. The αA-CRYBP1 protein also appears to be cleaved in a tissue-specific fashion. The mouse DE1 site appears to bind a member of the CREB/ATF family. Four functional elements (αBE-1, αBE-2, αBE-3, and MRF) have been identified in the mouse αB-crystallin enhancer; αBE-1, αBE-2, and αBE-3 are used in muscle and lens, while MRF binds myoD and myogenin and is muscle specific. We have identified in the chicken βA3/A1-crystallin gene an enhancer containing an AP-1 consensus-binding sequence which increases lens transcription but is not necessary for lens specificity. Transfection and gel mobility shift experiments indicate that the PL-1 and PL-2 functional elements of the chicken βB1-crystallin promoter and the AP-1/ARE sequence of two squid crystallin promoters are necessary for activity in transfected chicken lens cells and bind similar nuclear proteins of the chicken lens. Six chicken nuclear proteins that bind to the PL-1 sequence have been cloned. Transgenic mouse experiments indicate that the δ2-crystallin enhancer works efficiently in the lens and also has modest activity in the cornea, brain, and retina. Squid glutathione S-transferase (GST) and two squid S-crystallin cDNAs have been expressed; GST is very active while the S-crystallins show little if any activity. Cephalopod cDNAs for Ω-crystallin/ALDH, intermediate filament protein, and α- and β-tubulin have been cloned; the genes for all but α-tubulin were lens specific. Cloned cubomedusan jellyfish J3-crystallin has been shown to be a novel protein.

Project Description

Additional Personnel

Cynthia Jaworski	Ph.D.	Staff Fellow, LMDB, NEI
Marc Kantorow	Ph.D.	IRTA, LMDB, NEI
Xuan Li	Ph.D.	Visiting Associate, LMDB, NEI
Joan B. McDermott	M.S.	Biologist, LMDB, NEI
Barbara Norman		Chemist, LMDB, NEI
Christina M. Sax	Ph.D.	Senior Staff Fellow, LMDB, NEI
Stanislav I. Tomarev	Ph.D.	Visiting Scientist, LMDB, NEI

Objectives

The objective of this project is to understand the structure, organization, expression, and evolution of the gene families encoding the lens crystallins in the animal kingdom. Particular attention is given to the regulation of crystallin gene expression in the developing lens and, in the case of multifunctional crystallins and enzyme-crystallins, in nonlens tissues.

Methods

Conventional methods used for analysis of proteins and nucleic acids include polyacrylamide and agarose gel electrophoresis, RNA and DNA isolation, molecular hybridization (Southern and Northern blots), cDNA and gene cloning, DNA sequencing, recombinant DNA construction and mutagenesis, *in situ* hybridization, expression of recombinant DNAs in transfected cells and transgenic mice, polymerase chain reactions, primer extension and S1 protection experiments, *in vitro* and *in vivo* footprinting, gel mobility shift analysis, chromatographic purification of proteins, and Western immunoblotting.

Major Findings

α-crystallin.—There are two α-crystallin genes, αA and αB. Although both are expressed principally in the lens, the αB gene is expressed constitutively in many other tissues and is inducible by stress. By contrast, there is very little expression of αA in other tissues (although there is some), and it is not inducible by stress. We have been continuing our studies on the molecular basis for expression of the mouse and chicken αA and the mouse αB-crystallin genes.

At least four separate control sequences have been identified for the mouse αA-crystallin gene: DE1 (−111 to −97), αA-CRYBP1 region (−75 to −48), TATA/PE1 (−35 to −19), and PE2 (+24 to +43). Our current evidence suggests that the DE1 site is a cyclic AMP-responsive element (CRE) that binds a member of the CREB/ATF family of transcription factors. PE2 contains both an AP1 and a glucocorticoid-responsive element. The αA-CRYBP1 site binds a ubiquitous protein that we have cloned and called αA-CRYBP1. However, this site also contains a consensus sequence for the transcription factor, NF-κB.

Immunoblotting experiments indicate that the αA-CRYBP1 site binds various tissue-specific forms of the αA-CRYBP1 protein. Although there is no evidence indicating that NF-κB is used as a transcription factor for the expression of the mouse αA-crystallin gene, this cannot be ruled out at the present time. The αA-CRYBP1 gene has been cloned and shown to consist of at least seven exons. Its entire cDNA sequence is almost completed, except for a small stretch in the middle. The encoded protein contains at least four zinc fingers and is approximately 300,000 Daltons (D). It is homologous to the human PRDII-BF1/MBP transcription factor. Since αA-CRYBP1 is smaller than 300,000 D on Western blots, it appears as if the protein has been cleaved before use.

Sequencing multiple cDNAs has indicated that the primary transcript of αA-CRYBP1 is alternatively spliced, providing another possible basis for heterogeneity of this putative transcription factor. A series of mutated constructs inserted into transgenic mice have shown that the DE1 and αA-CRYBP1 sites are functionally redundant. It appears necessary for at least one of these control sites to interact with PE1 and/or PE2 for lens-specific expression to occur.

Work over the past 5 years has shown, surprisingly, that different control elements are used in expression of the orthologous mouse and chicken αA-crystallin genes. We have identified the following control sequences for the chicken gene: DE3 (−153 to −140), DE2A (−144 to −134; this overlaps with DE3), DE2B (−128 to −118), DE1A (−114 to −104), and DE1B (−100 to −93). The αA-CRYBP1 sequence in the chicken gene differs from that in the mouse gene by one nucleotide, and although it footprints with nuclear proteins from the chicken lens in DNAse I protection experiments, it does not

appear to be functional, as judged by mutagenesis and transfection tests. Three cDNAs encoding proteins that bind to the DE2A sequence have been cloned from an embryonic heart and are currently under investigation. In contrast to the DE1 region of the mouse αA-crystallin gene, the DE1A and DE1B sequences of the chicken αA gene do not appear to bind a member of the CREB/ATF family of transcription factors.

In 1991 we reported the presence of an enhancer at positions –426 to –259 of the αB-crystallin gene of the mouse. This sequence behaves as a strong enhancer for expression in cultured muscle cells and as a weak enhancer in cultured lens cells. We have now established by mutagenesis and footprinting experiments the presence of at least four functional elements within this enhancer: αBE-1 (–420 to –397), αBE-2 (–360 to –327), αBE-3 (–319 to –303), and the muscle regulatory factor (MRF) binding site (–300 to –280). The MRF contains an E box and is activated by the binding of either MyoD or myogenin.

Last year we indicated by DNAse I footprinting experiments that the –148/–118 sequence was necessary for the specific expression of the αB gene in the lens. This year transgenic mouse experiments have shown that this sequence is necessary for lens-specific expression but the enhancer is not. The –426/–259 enhancer sequence is necessary for expression of the αB gene in skeletal muscle and heart, and it quantitatively boosts expression in the lens. Thus, αBE-1, αBE-2, and αBE-3 are used for expression of the αB-crystallin gene in both lens and muscle cells, but the MRF element is used only in the muscle cells. This is the first example of a muscle-specific control element in a crystallin gene.

β-*crystallin.*—We have been investigating the βB1 and βA3/A1-crystallin genes in the chicken for several years. This year we have added the βB1-crystallin gene of the mouse to our studies. Previous experiments have established the importance of the PL-1 and PL-2 control elements for activity of the chicken βB1 promoter in transfection experiments. The PL-1 and PL-2 sequences are present between position –122 and the TATA box; they resemble AP-1 binding consensus sequences and compete with AP-1 sites for binding nuclear proteins.

This year we have selected six different cDNAs from an embryonic chicken heart library on the basis of binding to multimerized PL-1 sequences. They are currently under investigation. The –434/+30 sequence of the chicken βB1-crystallin gene has been shown to contain the information for lens specificity in transgenic mouse experiments. Deletion experiments are in progress to determine whether the PL-1 and PL-2 elements are necessary for lens specificity. Finally, we have cloned and are characterizing an approximately 18-kbp fragment that contains the mouse βB1-crystallin gene.

We are continuing our studies on the chicken βA3/A1-crystallin gene. The –287/–254 sequence has been shown to possess enhancer activity for expression in transfected embryonic chicken lens epithelial cells. It contains an AP-1 consensus sequence and binds multiple nuclear proteins in gel mobility shift experiments. A T-rich tract located between positions –218 and –168 appears to suppress activity of the –287/–254 enhancer in transfection experiments. A series of transgenic mice have been produced using the reporter gene chloramphenicol acetyltransferase (CAT). Fusion genes containing the –382/+22 or –143/+22 fragments of the βA3/A1 gene directed lens-specific expression, indicating that neither the –287/–254 enhancer nor the –218/–168 T tract is necessary for lens specificity.

δ-*crystallin.*—There are two linked δ-crystallin genes in the chicken. The 5′ gene is specialized for lens expression, while the 3′ gene encodes argininosuccinate lyase (ASL), making this an enzyme-crystallin. Although only the δ2 gene encodes a protein with ASL activity, both genes are expressed in a developmentally regulated fashion in the lens, cornea, retina, heart, and brain of the chicken embryo.

This year we generated transgenic mice carrying fusion genes comprising various combinations of the δ-crystallin promoters and enhancers (located in the third intron of the natural genes) attached to the bacterial CAT reporter gene. The enhancers of both δ-crystallin genes caused extremely high expression of the CAT gene in the lens of the transgenic mice, suggesting that a silencer for lens expression of the δ2-crystallin gene is operative in the chicken. Evidence also was obtained indicating that the δ-crystallin enhancers influence expression of the transgenes in the transgenic mouse cornea, retina, and brain in a way that is consistent with the expression of the natural genes in these tissues in the embryonic chicken.

The δ-crystallin locus has been cloned from the duck genome to compare the regulatory sequences

for these two genes in the duck and the chicken. We have undertaken this project because, in contrast to the chicken δ2 gene, the duck δ2-crystallin gene is very active in the lens. It should be able to help us understand the mechanism of suppression of the δ2 enhancer in the chicken lens. So far, we have established that the duck δ-crystallin genes are linked as in the chicken; further characterization is in progress.

S-crystallin.—Although much research had been performed on the lens crystallins of vertebrates, very little information was available concerning the major lens proteins of the complex eyes of invertebrates. Thus, several years ago we began studying invertebrate crystallins. Particular attention has been given to the crystallins of cephalopods (squids and octopi) because these species have the prototypical cellular invertebrate lenses that have evolved independent of those of vertebrates. We showed earlier that S-crystallins are encoded by a family of at least 10 genes that are expressed specifically in the lens and are related to the glutathione S-transferase (GST) genes of vertebrates.

Last year we cloned the squid gene encoding GST. In contrast to the S-crystallin genes, the GST gene is expressed principally in the digestive gland (analogous to the vertebrate liver) and very little in the lens or other tissues of the squid. This year we have expressed the cDNAs for the GST gene and for a minor (SL11) and major (SL20-1) S-crystallin gene of the squid in a bacterial extract and assayed for GST activity. The results showed that squid GST has more enzymatic activity than any other invertebrate or vertebrate GST ever reported. The major SL20-1 crystallin had essentially no GST activity, and the minor SL11 crystallin had slight GST activity. Thus, the S-crystallins generally lost GST activity as they specialized for expression in the lens. Interestingly, the loss of GST activity of SL20-1 is associated with the insertion of a novel peptide in the center of the protein. There is no insertion in the SL11 protein, which has some GST activity. Mutagenesis experiments are in progress to identify the active sites for substrate binding and for enzymatic function.

Transfection experiments using the CAT reporter gene and embryonic chicken lens epithelial cells have been conducted in order to identify putative control elements of the S-crystallin genes. An overlapping AP-1/antioxidant responsive element (ARE), present just upstream of the TATA box of the SL20-1 and SL11 crystallin genes of the squid, is required for promoter activity in the transfected chicken lens cells. A similar sequence is present in the PL-1 and PL-2 functional elements of the chicken βB1-crystallin gene. Gel mobility shift and competition experiments have provided evidence that the chicken PL-1 and PL-2 and squid AP-1/ARE regulatory sequences bind similar nuclear proteins of the chicken lens. These data raise the possibility that entirely different, nonhomologous crystallin genes of the chicken and squid have convergently evolved a similar *cis*-acting regulatory element for high expression in the lens. It is especially interesting that this element is a stress-responsive gene regulatory element, providing a further link between crystallins and stress proteins.

Ω-crystallin.—In addition to the major S-crystallins of cephalopods, a minor crystallin, called Ω-crystallin, is related to aldehyde dehydrogenase (ALDH). This is the only known invertebrate crystallin that has a vertebrate counterpart (i.e., η-crystallin/ALDH found in the elephant shrew). Last year we cloned Ω-crystallin cDNA. This year we finished characterizing the cDNA and the expression pattern of the Ω-crystallin gene. Sequence comparisons have suggested that vertebrate ALDH1/ALDH2 gene duplication occurred after the divergence of cephalopods from the line giving rise to vertebrates but before the separation of squid and octopus.

Southern blots are consistent with the presence of few, possibly only one, gene for Ω-crystallin in octopus and squid, and Northern blots indicate that this gene is expressed specifically in the lens. However, it is of particular interest that Ω-crystallin is the dominant protein in the muscle-derived, cellular lens of the ventral light organ in one squid (i.e., *Euprymna scolopes*). This indicates that the same gene has been recruited to be a crystallin in two entirely different lenses developing from different tissues. No ALDH activity has been found for Ω-crystallin. These results are consistent with the idea that, like the S-crystallins, Ω-crystallin evolved by duplication of an ancestral gene encoding ALDH and subsequently specialized for refraction in the transparent lens while losing enzymatic activity and expression in other tissues.

J-crystallin.—In addition to the cephalopod crystallins, we have been studying the crystallins of cubomedusan jellyfish, which also have complex

eyes with cellular lenses. We discovered several years ago that these lenses contain three apparently unrelated crystallins (J1, J2, and J3). Last year we cloned three genes encoding J1-crystallin polypeptides and showed that they lack introns and encode novel proteins. This year we have initiated studies on the J2- and J3-crystallins. Sequences of tryptic peptides have indicated that the J2 and J3 polypeptides are different proteins, despite their similarity in molecular mass (20 and 19 kD, respectively). A J3-crystallin cDNA has been cloned and partially sequenced. Like J1, J3-crystallin appears to be a novel protein; no homolog is reported in the data base. Interestingly, although the 19-kD J3 polypeptide is considerably smaller than the 35-kD J1 polypeptides, Northern blots indicate that the J3 mRNA is approximately 1.4 kb in length rather than the 1 kb size of the J1 mRNAs. We are now cloning the J3 gene(s).

Cytoskeletal proteins.—This year we completed analysis, initiated last year, of the intermediate filament (IF) protein and tubulin cDNAs of cephalopod lenses. Northern blots show that α-tubulin mRNA is present in all tissues examined, while the β-tubulin and IF mRNAs are lens specific. The proteins encoded in the tubulin cDNA sequences are very similar (87-93% identical) to the corresponding tubulins of insects and vertebrates, as expected for the high degree of conservation among these cytoskeletal proteins. In the IF protein, the central rod region is more highly conserved than the head and tail regions, yet even the rod region shows at most 39% identity with any other known rod region of an IF protein, namely with that of the squid neuronal IF protein. The rod regions of the squid lens IF protein contained the six heptads characteristic of nuclear lamins, consistent with an evolutionary relationship between IF proteins and lamins.

We had previously investigated the regulatory elements of the chicken vimentin gene because it is expressed relatively highly in the lens. Earlier transfection experiments revealed the presence of both positive- and negative-acting sequence elements within the first 767 nucleotides of the 5′-flanking region of the gene. This year we identified a silencer between positions −1360 and −1156 and an activator between positions −1612 and −1360 of the chicken vimentin gene. These regions, which contain numerous consensus sequences for the binding of transcription factors implicated in the expression of different crystallin genes, deserve further study.

Significance to Biomedical Research and the Program of the Institute

The crystallins comprise a diverse family of differentially expressed proteins that are required for the optical properties of the transparent lens. Understanding the structure, function, and evolution of these protein families and their genes contributes to our knowledge of embryonic development, eukaryotic gene expression, cell differentiation, molecular evolution, the visual system, and cataract. That crystallins are multifunctional proteins expressed in lens and nonlens tissues adds another dimension of interest and has implications for metabolism, cell biology, and drug and gene therapy.

Proposed Course

The following studies are proposed for Fiscal Year 1994:

1. We will continue identifying *cis*-acting elements in crystallin genes by mutagenesis and expression studies.

2. We will investigate the interaction of *cis* elements of the crystallin genes by footprinting and function studies.

3. We will continue cloning and characterizing putative transcription factors for crystallin genes by binding studies.

4. We will complete the sequences of the αA-CRYBP1 cDNA and gene in the mouse.

5. We will continue mutagenesis studies of the squid GST and S-crystallin cDNA to relate structurally the enzymatic and refractive functions of these proteins.

6. We will continue cloning and characterizing the jellyfish crystallin genes.

7. We will investigate the nature of the noncrystallin functions of the α-crystallin polypeptides.

8. We will continue investigations on the similarities and differences of the IF proteins of squid and vertebrates by conducting structural and function studies.

NEI Research Program

Lens and Cataract—Molecular Biology

Publications

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Zinovieva RD, Tomarev SI, Piatigorsky J: Aldehyde dehydrogenase-derived Ω-crystallins of squid and octopus. Specialization for lens expression. *J Biol Chem* 268:11449-11455, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00259-04 LMDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of the Cornea

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Joram Piatigorsky	Ph.D.	Chief	LMDB, NEI
Others:	W. Todd Kayes	Ph.D.	IRTA	LMDB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Molecular Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In the past 2 years we have cloned a number of abundant cDNAs from the corneal epithelial cells of the mouse. Some of these encode metabolic enzymes, suggesting that enzymes may act as crystallins in the transparent cornea as they do in the lens. Indeed we have found that different species accumulate different enzymes in their corneal epithelial cells, reminiscent of the taxon specificity observed for enzyme-crystallins of the lens. Aldehyde dehydrogenase (ALDH) class III is the major protein in the corneal epithelial cells of mammals. We have employed the RACE technique to clone the 5′ end of the ALDH class 3 corneal mRNA, identified the sequences that should constitute the 5′ exon of the gene, and cloned the ALDH class 3 gene in an 18 kbp genomic fragment. This fragment is undergoing analysis.

Project Description

Objectives

The project's objectives are to identify and characterize the genes that are preferentially expressed in the epithelium and endothelium of the cornea and to study the molecular basis for their expression in this transparent structure.

Methods

Conventional molecular biology methods of cloning, sequencing, recombinant DNA construction, transfection, and transgenic mouse production are used.

Major Findings

Aldehyde dehydrogenase (ALDH) class 3 comprises approximately 40% of the soluble protein of the corneal epithelial cells of the mouse and other mammals. Such abundance for a metabolic enzyme suggests that this protein is acting like an enzyme-crystallin and has a refractive function in the cornea as crystallins do in the lens. Last year we reported the cloning of the mouse ALDH gene. We thought at that time that we had approximately 2 kbp of the 5′-flanking region as well as the structural gene. Thus, we constructed a β-galactosidase fusion gene with what we believed was 1.1 kbp of the ALDH 5′-flanking sequence and used this construct as a transgene in transgenic mice.

Analysis of the progeny of these mice during this fiscal year showed that the transgene had been incorporated but that it was not expressed in any tissue, not even the cornea. We thus extracted more corneal RNA from mice and employed the RACE technique to clone the 5′ end of the ALDH cDNA. The sequence of the resulting clones showed that there is an additional exon 5′ beyond what we had cloned, implying that our β-galactosidase fusion gene lacked the ALDH promoter and other regulatory sequences for expression. Consequently the ALDH gene was cloned from a mouse genomic library. The 18-kbp, cloned DNA is now undergoing analysis.

Significance to Biomedical Research and the Program of the Institute

The molecular biology of corneal epithelium and endothelium has not advanced to the same extent as that of the collagenous stroma; consequently, it should be investigated. The cornea is a transparent, ectodermally derived tissue like the lens; thus comparative studies between it and the lens are of special interest with respect to transparency. Moreover, because of our finding that corneal epithelial cells show taxon-specific gene sharing of metabolic enzymes as does the lens, our major tissue of research, comparative studies on the cornea and lens, is of obvious importance from developmental and evolutionary viewpoints. Finally, the cornea is particularly accessible for gene therapy on account of its exposure to the surface and its association with numerous hereditary diseases.

Proposed Course

In Fiscal Year 1994 we will (1) analyze and sequence the mouse ALDH class 3 gene expressed in the cornea, (2) identify the promoter and functional regulatory elements for the ALDH class 3 gene by transfection experiments, and (3) create and analyze transgenic mice containing various truncated 5′-flanking sequences of the ALDH class 3 gene to identify the sequences responsible for high expression in the corneal epithelial cells.

NEI Research Program

Lens and Cataract—Molecular Biology

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00255-05 LMDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology and Functions of Lens Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Graeme J. Wistow	Ph.D.	Chief, Section on Molecular Structure and Function	LMDB, NEI
Others:	Vishwas Paralkar	Ph.D.	Visiting Associate	LMDB, NEI
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	Lorenzo Segovia	Ph.D.	Visiting Fellow	LMDB, NEI
	Jill Richardson	Ph.D.	Visiting Fellow	LMDB, NEI
	Cynthia Jaworski	Ph.D.	Chemist, Section on Molecular Genetics	LMDB, NEI
	Peggy Zelenka	Ph.D.	Chief, Section on Cellular Differentiation	LMDB, NEI

COOPERATING UNITS *(if any)*

DNX, Inc., Princeton, NJ

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Molecular Structure and Function

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.8	4.8	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Crystallins are stress-related proteins or enzymes that often maintain dual roles without gene duplication. We have shown that the putative lens promoter of NADPH: quinone oxidoreductase/ζ-crystallin is highly lens specific and by itself is responsible for the recruitment of this gene as a crystallin. An important functional element (ZPE) that binds different complexes in lens and nonlens-cell extracts has been identified. The full sequence of η-crystallin, another mammalian enzyme crystallin, has been obtained and shows identity with the enzyme ALDH1, another example of gene recruitment. μ-Crystallin, originally discovered in marsupial lenses, is a novel enzyme with NADPH-binding activity. Immunohistochemistry suggests it is preferentially expressed in vertebrate photoreceptors. The gene for μ has been cloned.

Not all important lens proteins are crystallins. We have shown that MIF, a lymphokine, is expressed in differentiating lens cells. The gene for human MIF has been cloned, and expression studies are under way. Lens P2 protein, another potential marker for the differentiation process and a possible intermediary messenger has been cloned and shown to belong to a lipid/retinoid-binding family. Members of the C/EBP family, transcription factors with important roles in differentiation and candidates for promoter binding in some crystallin genes, have been detected in the lens, where their pattern of expression is developmentally regulated.

Project Description

Objectives

We are investigating the molecular basis for normal lens structure and function, including the characterization of crystallins and the mechanisms of their normal expression. This also has led to the identification of molecules, such as transcription and growth factors, involved in lens cell differentiation. The interplay of such factors is an essential part of normal lens development and function.

Methods

A wide range of molecular biology techniques are used, including RNA analysis, gene and cDNA cloning and sequencing, functional gene promoter analysis in cultured cells and in transgenic mice, and polymerase chain reaction (PCR). We perform some protein analysis and make use of commercial facilities for protein sequencing.

Major Findings

Gene recruitment: Enzyme crystallins.—A novel enzyme with quinone reductase activity has undergone gene recruitment in certain mammals, acquiring a second function as the lens structural protein ζ-crystallin. In work by Drs. Douglas Lee and Jill Richardson, we have shown that recruitment of this taxon-specific crystallin can be explained by the lens specificity of an alternative promoter. By itself this promoter confers strongly lens-preferred expression in both cultured cells and transgenic mice.

While proximal regions of the promoter have some activity in the transgenic brain, this is abolished by the addition of more distal regions. The minimal active promoter contains a region, ZPE (ζ-protected element), including a consensus C/EBP binding site, which is strongly and identically footprinted by nuclear extracts from both mouse and rabbit cultured lens cells in which the promoter is active. In fibroblast nuclear extract, the ZPE's more restricted footprint is flanked by other protected regions that are absent or only weakly footprinted in lens cell extracts. In gel shift assays, the ZPE sequence forms specific complexes with lens-cell extracts but not with fibroblast extracts. Additional transgenic mice have been generated and show that sequences between positions −385 and −533 are required for suppression of promoter expression in the brain.

μ-Crystallin is the major component of the eye lens in several Australian marsupials. It also is a novel enzyme in other mammals, including humans, where it has nonlens expression in neural tissue (including retina), muscle, and kidney. It has striking sequence similarity with bacterial ornithine cyclodeaminases, suggesting an unusual role in ornithine metabolism. Dr. Lorenzo Segovia has shown that μ-crystallin preferentially binds NADPH, consistent with its role as a reductase. Immunohistochemistry of the developing rat shows a remarkable, intense reaction with retinal photoreceptors; in fact, our anti-μ antiserum detects the earliest marker known for photoreceptor cells. Since the retina is susceptible to ornithine toxicity in gyrate atrophy, the expression of a novel ornithine-metabolizing enzyme in photoreceptors could be significant. The kangaroo gene for this enzyme crystallin has been cloned and is being analyzed.

Another major enzyme crystallin (up to 25% of total protein) in mammals is η-crystallin found in elephant shrews. Ms. Caroline Graham has cloned the complete cDNA sequence for this protein from two different species. As predicted, sequence data confirm that η-crystallin is ALDH1 and that it is the product of a single recruited gene. cDNA clones will be expressed in bacterial hosts to characterize enzyme activity.

Dr. Cynthia Jaworski has completed the cDNA sequence of human αA-crystallin and has thereby corrected sequence errors in older protein data. αA-crystallin is the single major component of the human lens and is related to small heat shock proteins (shsp). The quaternary structure of α-crystallins and shsps is both controversial and of great interest. New model structures were predicted on the basis of existing biochemical data. Briefly, these correspond to cubic and dodecahedral structures with conserved intermolecular contacts. These predictions are stimulating biophysical investigations in other laboratories.

αB-crystallin is multifunctional, serving as both a major structural protein in the lens and as an shsp in other tissues in mammals. By cloning and Northern analysis, Dr. Lee showed similarly that αB-crystallin mRNA is present in all the mature tissues examined in a bird (*Anas platyrhynchos*), although

there are some differences in the pattern of transcripts seen. Interestingly, sequence analysis not only shows that duck αB-crystallin is a member of the shsp family, as expected, but that this family shares more distant similarity with another heat shock protein (hsp) family, the highly conserved HSP70s of both eukaryotes and prokaryotes. This raises the interesting possibility that large and small hsps may share structural and perhaps functional features.

Molecular markers of differentiation.—Macrophage migration inhibitory factor (MIF) was originally identified as a lymphokine. However, recent work strongly suggests a role for MIF beyond the immune system. Expressed specifically in the differentiating cells of the immunologically privileged eye lens and brain, it is a delayed early response gene in fibroblasts but is expressed in many tissues. In contrast to previous reports, we have found evidence for a single gene for MIF in the human genome. Dr. Vishwas Paralkar has shown that this small gene has three exons separated by introns of only 189 and 95 bp and covers less than 1 kb. The cloned sequence also includes 1 kb of 5′-flanking region covering the putative promoter.

Primer extension and 5′ RACE PCR of human brain RNA both indicate the presence of a single transcription start site in a TATA-less promoter. Northern blot analysis shows a single-size MIF mRNA in all human tissues examined. MIF mRNA is particularly abundant in the kidney and is expressed at high levels in many other tissues but is at low levels in muscle and the pancreas. The relatively abundant expression of MIF in lens may have clinical significance, with the possibility of involvement in lens-induced endophthalmitis and uveitis.

Another potential differentiation marker also was discovered by protein microsequencing of a 14-kD band in calf lens extract. Dr. Jaworski has now obtained the complete cDNA sequence for this protein by PCR methods. The protein is a member of the lipid/retinoic acid-binding family of P2 proteins. Since retinoic acid has now been implicated in γ-crystallin gene expression, this protein could have a direct role in mediating lens gene expression.

We are interested in connections between differentiation in the well-studied adipocyte system and in the eye lens. In both systems there is a switch in c-myc expression during differentiation: Stem cells are marked by expression of α-enolase while P2-like proteins also may be associated with differentiation. Furthermore, C/EBP-like proteins are candidates for binding to an essential, tissue-specific region of the ζ-crystallin gene lens promoter. Dr. Richardson is examining whether C/EBP-like proteins are expressed in the lens. She has results from immunohistochemistry associating expression of C/EBP β and δ with rat lens epithelia, the relatively undifferentiated "stem cell" population. C/EBP δ is most abundant in the central, quiescent cells, while β comes on downstream in regions where cells are migrating and dividing. Both are undetectable in the terminally differentiated lens fiber cells.

The human Marner cataract maps to chromosome 16 near the locus of several cadherins, which are important cell adhesion molecules. The cataract manifests as opacities at the Y-sutures, suggesting that a cell-cell contact or adhesion process could be defective. We examined whether a cadherin could be involved in this cataract. Immunochemical methods suggest that N-cadherin is expressed in the eye lens, but having no sequence data, this could be a cross-reaction with a related, perhaps lens-specific, variant.

Ms. Graham used PCR to amplify cadherin-related sequences in human fetal lens and sequenced multiple clones. All were identical to known human N-cadherin. We then checked the chromosomal location of N-cadherin by PCR, using chromosome-specific template DNA, confirming its published position on chromosome 18. It is the only major cadherin not on chromosome 16.

These results suggest that the principal cadherin expressed in the eye lens is identical to N-cadherin and that there is no lens-specific variant of this family. The only remaining possibilities of a connection between cadherins and the Marner cataract are that another cadherin is expressed at low levels in the lens or with a particular development pattern, or that the mutation causes inappropriate expression in the lens of another cadherin.

Significance to Biomedical Research and the Program of the Institute

The discovery of fundamental mechanisms in the differentiation and evolution of complex tissues has had important results in our understanding of important processes in evolution and in tissue-specific expression. In the process, we have discovered a novel enzyme that has possible significance in the

human retina. We also have discovered important markers for cellular differentiation, including proteins with inflammation-related lymphokine activity.

Proposed Course

1. We will continue examining the molecular mechanisms for lens-preferred expression and for gene recruitment in ζ, η, μ, and τ-crystallins.

2. We will characterize the function and nonlens role of μ-crystallin.

3. We will explore the molecular biology and function of MIF expressed in the lens and its possible role in eye inflammation.

4. We will determine the function and pattern of expression of the lens P2 protein, a possible second messenger in signal transduction.

NEI Research Program

Cataract—Molecular Genetics

Publications

Chen H, Phillips HA, Callen DF, Kim RY, Wistow GJ, Antonarakis SE: Localization of the human gene for mu-crystallin to chromosome 16p. *Genomics* 14:1115-1116, 1992.

de Jong WW, Leunissen JAM, Wistow GJ: Eye lens crystallins and the phylogeny of placental orders: Evidence for a macroscelid-paenungulate clade? in Szalay FS, Novacek MJ, McKenna MC (eds): *Mammal Phylogeny: Placentals.* New York, Springer Verlag, 1992, pp 5-12.

Hodin J, Wistow G: 5′-RACE PCR of mRNA for three taxon-specific crystallins: For each gene one promoter controls both lens and non-lens expression. *Biochem Biophys Res Commun* 190:391-396, 1993.

Kim RY, Gasser R, Wistow GJ: Mu-crystallin is a mammalian homologue of Agrobacterium ornithine cyclodeaminase and is expressed in human retina. *Proc Natl Acad Sci USA* 89:9292-9296, 1992.

Kim RY, Wistow GJ: The cDNA RPE1 and monoclonal antibody HMB-50 define gene products preferentially expressed in retinal pigment epithelium. *Exp Eye Res* 55:657-662, 1992.

Kim RY, Wistow GJ: Expression of the duck α-enolase/τ-crystallin gene in transgenic mice. *FASEB J* 7:464-469, 1993.

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Lee DC, Kim RY, Wistow GJ: An avian αB-crystallin. Non-lens expression and sequence similarities with both small (HSP27) and large (HSP70) heat shock proteins. *J Mol Biol* 232:1221-1226, 1993.

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Wistow G: Possible tetramer-based quaternary structures for α-crystallins and small heat-shock proteins. *Exp Eye Res* 56:729-732, 1993.

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Wistow GJ, Shaughnessy MP, Lee DC, Hodin J, Zelenka PS: A macrophage migration inhibitory factor is expressed in the differentiating cells of the eye lens. *Proc Natl Acad Sci USA* 90:1272-1275, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00251-06 LMDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetically Engineering the Eye with the αA-Crystallin Promoter

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ana B. Chepelinsky	Ph.D.	Head, Section on Regulation of Gene Expression	LMDB, NEI
Others:	Devonne M. Parker	B.S.	Biologist	LMDB, NEI

COOPERATING UNITS *(if any)*

Department of Cell Biology, Baylor College of Medicine, Howard Hughes Medical Institute (Paul Overbeek, Ph.D.; Michael Robinson, Ph.D.); Imperial Cancer Research Fund, London, England (Clive Dickson, Ph.D.); Gerontological Research Unit, National Institute of Health and Medical Research, Paris, France (Yves Courtois, Ph.D.; Maryvonne Laurent, Ph.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Regulation of Gene Expression

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.9	0.4	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Gamma interferon (IFN-γ) expression was directed to the eyes of transgenic mice to investigate the possible role of this lymphokine in ocular pathogenesis. The most notable effects of IFN-γ in these transgenic mice include microphthalmia, blepharophimosis, microphakia, impairment of lens fiber formation, arrest of retinal differentiation, retinal detachment, and persistent hyperplastic primary vitreous. Major histocompatibility complex (MHC) class II mRNA levels were significantly increased in the transgenic eyes, and MHC class II proteins were expressed in the cornea, iris, ciliary body, choroid, lens, and retinal pigment epithelium.

Int-2/fibroblast growth factor (FGF)-3 expression was directed to the eye to investigate how the aberrant expression of this growth factor would affect the developmental program of the eye. The ectopic expression of int-2 during the embryonic development of the lens affected the differentiation of the entire eye, highlighted by the appearance of intraocular secretory glandular epithelium, similar to dermoid-like pathology.

Project Description

Additional Personnel

Charles Egwuagu	Ph.D.	Scientist, Public Health Service, LI, NEI
Chi-Chao Chan	M.D.	Chief, Section on Immunopathology, LI, NEI
Robert B. Nussenblatt	M.D.	Clinical Director, LI, NEI
Jorge Sztein	D.V.M.	Visiting Associate, Veterinary Research Resources Service, NEI

Objectives

The objective of this project is to understand how aberrant genetic expression of interferon gamma (IFN-γ), int-2, or acidic fibroblast growth factor (aFGF), under the control of the αA-crystallin promoter, perturbs normal eye development in transgenic mice.

Methods

Recombinant DNA techniques used in this study include plasmid construction, oligonucleotide sequencing, Southern and Northern hybridizations, DNA sequencing, primer extension, polymerase chain reaction (PCR), reversed transcription PCR, immunohistochemistry, and the production and analysis of transgenic mice.

Major Findings

IFN-γ.—This project is conducted in collaboration with Drs. Charles Egwuagu, Jorge Sztein, Chi-Chao Chan, and Robert B. Nussenblatt from the NEI Laboratory of Immunology. The aberrant expression of IFN-γ in the lens of transgenic mice allowed us to study the effect of IFN-γ on the normal development of the eye and the regulation of major histocompatibility complex (MHC) class II gene expression by IFN-γ in a nonlymphoid tissue such as the lens.

We generated transgenic mice containing as a transgene the murine αA-crystallin promoter (−366/+46) fused to the murine IFN-γ coding sequence. The ectopic expression of IFN-γ in the lens of the transgenic mouse affected the growth of the whole eye, resulting in microphthalmia and microphakia. The lens fiber cells were replaced by balloon cells, differentiation of the neuroretina into inner and outer neuroblastic layers was arrested at the embryonic stage, and retinal detachment and the presence of macrophages in the subretinal space were observed in the adult mouse eye.

Constitutive expression of IFN-γ in the lens induced aberrant MHC class II protein synthesis in several ocular tissues. This transgenic mouse serves as an animal model to (1) facilitate understanding of the molecular pathways governing synchronized programmed differentiation of ocular tissues and (2) enable study of the linkage between aberrant MHC class II expression and predisposition to autoimmune diseases.

int-2.—This project is conducted in collaboration with Drs. Paul Overbeek and Michael Robinson (Baylor College of Medicine) and Dr. Clive Dickson (Imperial Cancer Research Fund). To assess whether ectopic expression of the proto-oncogene int-2/FGF-3 would perturb normal eye development, we directed its expression to the eyes of transgenic mice with the murine αA-crystallin promoter. We obtained three lines of transgenic mice expressing the αA-crystallin/int-2 transgene. These adult transgenic mice presented microphthalmia characterized by intraocular hyperplastic glandular structures replacing the normal iris, ciliary body, and lens; the retinas showed various degrees of dysplasia.

The intraocular glandular structures of these mice stained positively for int-2 and Muc-1, a marker for secretory epithelia. We also observed a marked increase in Muc-1 mRNA levels and a drastic decrease in MIP (major intrinsic protein) mRNA levels, a marker of lens fibers, in the eyes of the adult transgenic mice. Proptosis of the transgenic eye, observed as early as day 15 of embryonic development, was followed by expulsion of the lens through the cornea and detachment of the undifferentiated retina. The newborn mice presented a "scabby eye" phenotype. Ectopic expression of the growth factor int-2 during the embryonic development of the lens affected the differentiation of the entire eye, highlighted by the appearance of intraocular secretory glandular epithelium, similar to dermoid-like pathology, in the adult eye.

aFGF.—In collaboration with Drs. Overbeek and Robinson , as well as Dr. Yves Courtois (Institute for Gerontological Research, INSERM), we injected into mouse embryos a recombinant DNA containing the αA-crystallin promoter (−366/+46) fused to the bovine aFGF cDNA. Three lines of transgenic mice

were obtained; no particular phenotype was observed. We currently are analyzing these mice for expression of the transgene.

Significance to Biomedical Research and the Program of the Institute

The aberrant expression of IFN-γ or int-2 will allow us to elucidate the mechanisms underlying eye development. At the same time, it opens new avenues in the development of animal models for studies of eye pathologies and of gene regulation in the eye.

Proposed Course

The following studies will continue during Fiscal Year 1994:

1. We will characterize further the effect of IFN-γ on the regulation of gene expression in the eyes of the transgenic mice.

2. We will continue to study the effect of int-2 on gene expression in the eyes of the transgenic mice and try to understand its role in eye growth and differentiation.

NEI Research Program

Cataract—Molecular Genetics

Publications

Chepelinsky AB, Overbeek PA, Chan C-C, Jamieson S, Dickson C, Parker DM, Robinson M: Int-2 ectopic expression induces differentiation of secretory epithelia in the eyes of transgenic mice. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1222, 1993.

Egwuagu CF, Sztein J, Chan C-C, Reid W, Mahdi R, Nussenblatt RB, Chepelinsky AB: Ectopic expression of gamma interferon in the eyes of transgenic mice induces ocular pathology and MHC class II gene expression. *Invest Ophthalmol Vis Sci*, in press.

Egwuagu CF, Sztein J, Chan C-C, Reid W, Mahdi R, Nussenblatt RB, Chepelinsky AB: Gamma interferon expression in the eyes of transgenic mice disrupts differentiation of the lens and retina. *Invest Ophthalmol Vis Sci* 34(4)(suppl): 1455, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00253-05 LMDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Expression of Lens Fiber Membrane Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ana B. Chepelinsky	Ph.D.	Head, Section on Regulation of Gene Expression	LMDB, NEI
Others:	Chiaki Ohtaka-Maruyama	Ph.D.	Visiting Fellow	LMDB, NEI
	Xiaoyan Wang	M.D.	IRTA Fellow	LMDB, NEI
	LaShawn R. Drew	B.S.	Chemist	LMDB, NEI
	Devonne M. Parker	B.S.	Biologist	LMDB, NEI

COOPERATING UNITS *(if any)*

Harvard University (David Paul, Ph.D.); Columbia University (Jorge Fischbarg, M.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Regulation of Gene Expression

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.2	2.0	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project studies the regulation of expression of genes encoding lens fiber membrane channel proteins. We are focusing on the regulation of expression of the gene encoding MIP, the major intrinsic protein of the lens fiber membrane, which is specifically expressed in the ocular lens fibers and belongs to an ancient superfamily of transmembrane channel proteins.

We characterized 2,840 bp of 5′ flanking sequence of the human MIP gene to study the *cis* regulatory elements responsible for the tissue specificity and developmental regulation of the MIP gene. We found that a DNA fragment containing 253 bp of 5′ flanking sequence and 42 bp of exon 1 of the human MIP gene fused to the reporter chloramphenicol acetyltransferase (CAT) gene is able to express the CAT gene in cultured lens cells. We are studying the interaction of transcription factors with the *cis* regulatory elements of the MIP gene and its effect on the *in vitro* transcription of the MIP gene in *Drosophila* nuclear extracts. Purified human Sp1 and AP2 interact with *cis* regulatory elements of the MIP promoter and activate the *in vitro* transcription of the MIP promoter, suggesting their involvement in the regulation of MIP gene transcription. These studies will further our understanding of the role of general transcription factors on the tissue-specific expression of the MIP gene.

Project Description

Objectives

The objective of this project is to elucidate the mechanisms involved in the regulation of expression of fiber membrane genes involved in cell-cell communication in the lens. The identification of the *cis* regulatory elements of these genes and their interaction with *trans*-acting factors is essential for understanding the regulation of gene expression in the lens.

Methods

Recombinant DNA techniques used in this study include screening genomic libraries, subcloning, plasmid construction, oligonucleotide synthesis, Southern and Northern hybridizations, DNA sequencing, primer extension, polymerase chain reaction (PCR), reversed transcription PCR, gel mobility shift assays, DNA footprinting, methylation interference, subcellular fractionation to obtain nuclear extracts, *in vitro* transcription, tissue culture techniques (including transfection of primary lens explants and cell lines), and analysis of transgenic mice.

Major Findings

Cis *regulatory elements of the human major intrinsic protein (MIP) gene promoter.*—We characterized 2,840 bp of 5′-flanking sequence of the human MIP gene to identify the *cis* regulatory elements responsible for the tissue specificity and developmental regulation of the MIP gene. We found that a DNA fragment containing 253 bp of 5′-flanking sequence and 42 bp of exon 1 of the human MIP gene fused to the reporter gene chloramphenicol acetyltransferase (CAT) directs CAT gene expression to lens cells in transient assays. Therefore, the −253/+42 sequence of the human MIP gene contains information for lens cell expression, suggesting that *cis* regulatory elements responsible for the lens-specific expression of the MIP gene are localized within this domain.

Several motifs known to bind transcription factors in other genes are present in the 5′-flanking sequence of the MIP gene. To elucidate whether those motifs are involved in the regulation of MIP gene expression, we are studying their interaction with several transcripton factors. Analysis by DNA footprinting and gel mobility shift assays show that purified human Sp1 and AP2 interact with several domains of the MIP promoter. They also activate the *in vitro* transcription of the MIP promoter in *Drosophila* nuclear extracts. We found that the initiation site of transcription of the human MIP gene was the same *in vivo* and *in vitro*. These results suggest that SP1 and AP2 are involved in the regulation of transcription of the MIP gene.

We have generated several lines of transgenic mice containing 253 bp of the human MIP gene 5′-flanking sequence with 42 bp of exon 1 fused to the CAT gene as a transgene. In one transgenic line, the CAT gene expressed specifically in the ocular lens. Expression of the CAT gene was observed in the ovaries of the females of four additional transgenic lines, although no expression was observed in any tissue of the male progeny. We currently are mapping other regulatory elements localized between −2,800 bp and the initiation site of transcription of the MIP gene that may be required for proper tissue specificity. We also are cloning the murine MIP gene in order to study the mouse MIP promoter in its homologous *in vivo* environment.

3′ untranslated sequence of the MIP gene.—We are sequencing the 3′-flanking region of the human MIP gene to characterize the polyadenylation sites and the processing of the two MIP transcripts observed *in vivo*.

Cloning of the connexin 46 gene.—We are cloning the connexin 46 gene, which encodes one of the lens fiber gap junction proteins, to be able to study how its expression is regulated in the lens.

CHIP28 expression in cornea endothelial cells.—In collaboration with Dr. Jorge Fischbarg (Columbia University), we are characterizing the member of the MIP family responsible for the CHIP28-like water channels observed in primary cultures of bovine cornea endothelial cells. Sequencing data indicate that it is CHIP28.

Significance to Biomedical Research and the Program of the Institute

Since the differentiation of lens epithelial cells into fiber cells results in a dramatic increase of new plasma membrane synthesis by elongating cells, proper membrane biosynthesis and physiology are of utmost importance in maintaining the transparent state of the lens. Membrane protein synthesis is regulated in a temporal and spatial manner in the lens. Alterations in lens membranes, particularly involving MIP, have been observed during cataractogenesis and aging. Therefore, studies on MIP gene

expression should further our understanding, not only of the mechanisms involved in the regulation of gene expression in the normal lens, but also of its disruption during disease.

Proposed Course

The following studies will continue during Fiscal Year 1994:

1. Characterization of the *cis* regulatory elements of the human MIP promoter.

2. Isolation of the murine MIP gene promoter and its comparison with its human homolog.

3. Study of the interaction of the MIP gene *cis* regulatory elements with transcription factors.

4. Characterization of the 3′-flanking region of the human MIP gene.

NEI Research Program

Cataract—Molecular Genetics

Publications

Chepelinsky AB: The MIP transmembrane channel family, in Peracchia C (ed): *Handbook of Membrane Channels: Molecular and Cellular Physiology.* Academic Press, in press.

Ohtaka-Maruyama C, Drew LR, Pisano MM, Chepelinsky AB: Regulatory elements of the MIP gene promoter. *Invest Ophthalmol Vis Sci* 34(4) (suppl):1342, 1993.

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Tomarev SI, Zinovieva RD, Weis VM, Chepelinsky AB, Piatigorsky J, McFall-Ngai MJ: Abundant mRNAs in the squid light organ encode proteins with a high similarity to mammalian peroxidases. *Gene* 132:219-226, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00285-01 LMDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

NEI Central Transgenic Animal Production Facility

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Eric Wawrousek	Ph.D.	Research Biologist	LMDB, NEI
Others:	Susan DiCamillo	B.S.	Chemist	LMDB, NEI
	R. Steven Lee	B.S.	Biologist	LMDB, NEI
	Mariana Gonzalez-Baez		Biological Science Lab Aide, Stay-in-School Program	LMDB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Transgenic Animal and Genome Manipulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.7	0.5	2.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The NEI Central Transgenic Animal Production Facility is a research support facility for all NEI intramural researchers requiring the use of transgenic mice in their research programs. We are providing transgenic animal support to 18 researchers from four laboratories in the NEI (Laboratory of Immunology, Laboratory of Mechanisms of Ocular Diseases, Laboratory of Molecular and Developmental Biology, and Laboratory of Retinal Cell and Molecular Biology); in our program, there are 52 DNA constructs at various stages of completion. NEI researchers using molecular biology techniques to study the eye submit DNA constructs to our section for production of transgenic mice. We create transgenic mice by standard procedures, then biopsy and perform DNA analysis on the mice born from these procedures to identify positive mice. At researchers' request, we mate positive transgenic mice, wean litters, biopsy and analyze DNA from successive generations of transgenic mice, and provide the transgenic animals to researchers for use in their experiments. Over the year we have generated 129 transgenic mice from 26 DNA constructs; set up 231 matings of transgenic mice; weaned, tagged, and tail-biopsied 3,033 mice; and isolated DNA and performed DNA analysis on 2,313 biopsy samples. We are working toward creating an embryo cryopreservation and banking program to provide long-term storage of important transgenic lines to eliminate the need to maintain live mice. In addition to service functions, we collaborate with NEI researchers on transgenic animal projects. This year we collaborated with Dr. Igal Gery (Laboratory of Immunology, NEI) in creating DNA constructs, and subsequently transgenic mice, to examine the immunologic consequences of expressing foreign antigens in the encapsulated ocular lens.

Project Description

Objectives

This project has been established to (1) produce transgenic animals for use in NEI's eye research, (2) supply ancillary services related to maintenance of transgenic animals, (3) provide advice and expertise in matters of transgenic animal projects to all NEI intramural researchers using this technology in their research, and (4) act as a central facility for all transgenic animal work conducted in the NEI Intramural Research Program in order to coordinate and conserve resources and utilize severely limited animal housing space with maximum efficiency. We provide a comprehensive program for short- and long-term storage of transgenic animal lines, both as live animals and as frozen embryos.

Methods

Standard methods are used for microinjecting DNA into the pronucleus of one-celled mouse embryos and surgically reimplanting the injected embryos into foster mothers for development. Conventional molecular biology techniques are used to isolate and analyze DNA from biopsy samples of transgenic mice. Data on all transgenic mice are maintained in a computerized relational data base accessed by programs written within our group.

Major Findings

Production of new transgenic mouse lines.—We have generated 129 new transgenic founder mice from 26 constructs submitted by researchers in 4 NEI intramural laboratories. These constructs are quite diverse in nature, reflecting the diversity of research being performed in the NEI. Some of the general categories of constructs are (1) promoter/reporter constructs in which the promoter of an eye gene is fused to a reporter gene to assess transcriptional activity in the transgenic mouse, (2) eye-specific or ubiquitous promoters driving expression of genes believed to be involved in eye pathologies to assess their roles in pathological conditions in a transgenic mouse model, and (3) other constructs for probing normal eye function and pathological conditions in the mouse.

Maintenance of transgenic mouse lines.—Transgenic mouse lines are derived by mating of the original transgenic founder mice and derivation of successive generations of progeny, which are then used in biomedical research. To generate lines of transgenic mice from our transgenic founder mice, we have set up 231 mouse matings and weaned, tagged, and biopsied 3,033 mice resulting from matings and microinjection procedures.

DNA analyses.—Approximately 10-25% of mice born from microinjected embryos are transgenic; similarly, approximately 50% of mice resulting from a transgenic mouse mating are transgenic. A rapid, efficient, and reliable method of identifying transgene positive and negative mice is in place in our group. We have processed 2,313 biopsy samples to obtain DNA and have performed analyses on these samples to determine whether the mice were transgene positive.

Embryo cryopreservation and banking.—We have been experimenting with freezing mouse embryos for banking of important lines of transgenic mice. Slow freezing of embryos in vials and in straws has been attempted. The straw method has the advantages of being simpler and more time efficient and will be pursued further. Reconstitution of mice has been accomplished by transferring thawed embryos into the oviduct or into the uterus of foster mothers. We have had the best results with oviductal transfers and will pursue this methodology. Our reproducibility in freezing and thawing embryos and in reconstituting mouse lines from thawed embryos is not yet sufficient to begin large-scale banking of embryos with confidence that we can regenerate the banked lines.

Significance to Biomedical Research and the Program of the Institute

Transgenic mice are currently the only readily attainable system for studying gene expression in the context of an entire, intact animal. While tissue culture can yield a great deal of information in many studies, true understanding of how a particular gene affects an organ (such as the eye) or an entire organism can be obtained only by studying that gene in the intact organism. We play a pivotal role in many NEI intramural research projects by providing the technology and expertise to insert into the mouse genes related to normal eye development and pathological eye conditions.

Proposed Course

In Fiscal Year 1994 we will continue our research as follows:

1. We will continue producing new transgenic mice for NEI researchers as required for their research projects.

2. We will continue breeding and maintaining the existing transgenic mouse lines needed for ongoing NEI research.

3. We will continue our efforts to reproducibly freeze and thaw mouse embryos and reconstitute mouse lines from the thawed embryos. Once we are certain that we can regenerate transgenic mouse lines from frozen embryos, we will begin cryopreservation and banking of some existing transgenic mouse lines, which are important to keep but which are not currently in use. This will free some of our limited animal housing space and ensure that important lines of mice will not be lost due to aging and loss of fertility.

NEI Research Program

Cataract—Molecular Genetics

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00286-01 LMDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

α-Crystallin Gene Disruption in the Mouse

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Eric Wawrousek Ph.D. Research Biologist LMDB, NEI

COOPERATING UNITS *(if any)*

University of Maryland Medical School (Nicholas Ambulos, Ph.D.)

LAB/BRANCH

Laboratory of Molecular and Developmental Biology

SECTION

Section on Transgenic Animal and Genome Manipulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The α-crystallins, which comprise a large fraction of the soluble protein in the vertebrate lens, where they are believed to function solely as structural proteins, are the first crystallins to be expressed in the developing mouse lens and are a relatively small family of crystallins encoded by only two genes, the αA- and αB-crystallin genes. The α-crystallins exhibit molecular chaperone activity and, at least in the case of αB-crystallin, have been shown to be expressed in a variety of nonlenticular tissues, where their function is unknown. Toward understanding the role of the α-crystallins in lens and nonlens tissues, we are attempting functional deletion of α-crystallins by disrupting the α-crystallin genes in mice. We are employing the technique of homologous recombination in pluripotent mouse embryonic stem cells, followed by the generation of chimeric mice containing the altered stem cells. We have isolated and mapped 15-kb clones containing the αA- and αB-crystallin gene loci from a mouse 129SV library (the same strain as most of the embryonic stem cell lines currently in use). Construction of the αA-crystallin "knockout vector" is near completion. In collaboration with Dr. Nicholas Ambulos (University of Maryland Medical School), we have nearly completed double-stranded sequencing of the mouse αA-crystallin gene (5 kb) and single-stranded sequencing of an additional 4 kb of the 5′-flanking sequence. We currently are generating constructs to look for enhancer regions in the αA-crystallin introns and in the 5′- and 3′-flanking regions.

Project Description

Additional Personnel

Ellen Liberman	Ph.D.	Division of Basic Vision Research, NEI

Objectives

This project was designed to disrupt the α-crystallin genes (αA and αB) in the mouse to study their effect on normal lens and eye development. Disruption of the genes essentially will delete these proteins from the mouse, enabling us to analyze the effects these proteins have on expression of other lens proteins, developmental regulation and morphology of the lens and other eye structures, and the role of these proteins in nonlenticular tissues.

Methods

Standard molecular biology techniques are used to clone the α-crystallin genes and construct "gene knockout" vectors. Disruption of the genes will be accomplished by the newly developed technology of homologous recombination in pluripotent mouse embryonic stem cells, followed by insertion of the genetically altered cells into blastocyst mouse embryos to generate chimeric mice with the gene disruption. Chimeric "knockout" mice will be bred to generate mice with heterozygous and homozygous knockouts.

Major Findings

Cloning of mouse α-crystallin genes.—To maximize the success of homologous recombination in the planned experiments, we have isolated clones for αA- and αB-crystallin from a mouse 129 SV genomic library. This is the mouse strain from which most currently used embryonic stem cells are derived. Use of isogeneic DNA has been shown to improve greatly the yield of correctly targeted gene disruptions. Two overlapping αA clones and five overlapping αB clones have been isolated and mapped. A 15-kb αA clone with 9 kb of 5′- and 2 kb of 3′-flanking sequence and a 16-kb αB clone with 7 kb of 5′- and 6 kb of 3′-flanking sequence will be used in construction of the knockout vector.

Construction of knockout vectors.—Construction of the αA-crystallin gene knockout vector is nearly complete. The vector contains 9 kb of αA 5′-flanking sequence, a selectable neomycin resistance gene cassette disrupting the αA gene, 1.3 kb of αA sequence, and a negative selectable marker (HSV tk).

Sequencing of mouse αA-crystallin gene.—We are now sequencing the mouse αA-crystallin gene locus in collaboration with Dr. Nicholas Ambulos. A double-stranded sequence of the gene is nearly complete, and a single-stranded sequence of 4 kb of 5′-flanking sequence has been done. Having the sequence of the locus will enable easy interpretation of DNA analysis of stem cells and mouse biopsies carrying the gene knockout. It also will facilitate identification of binding sites for regulatory protein in portions of the gene not yet studied.

Transcriptional regulation of mouse αA-crystallin gene.—We are constructing vectors containing portions of the αA-crystallin gene locus to search for transcriptional enhancer elements. A base vector containing the αA-crystallin promoter (–366 to +46) fused to the bacterial CAT reporter gene is under construction. Large pieces of the αA locus will be inserted into the base vector and tested for enhancer activity in transient transfection assays in cultured cells.

Significance to Biomedical Research and the Program of the Institute

Deletion of the α-crystallin proteins, individually or together, will provide a fundamental understanding of how these proteins function during normal lens development and how they may influence the structure and function of the lens and the entire eye. In addition, it would give us insight into the function of these proteins in nonlenticular tissues, which in turn could help us understand some of their more subtle roles in the eye.

Proposed Course

In Fiscal Year 1994 we will continue our investigation as follows:

1. We will continue construction of αA and αB knockout vectors so that mice deficient in either of these genes can be created. We also will perform the gene knockouts by introducing the targeting vector into mouse embryonic stem cells, selecting appropriately altered cells, and then creating chimeric mice from these cells. Deletion of a single allele of either αA or αB can be studied to assess the gene dosage effect (50% reduction of protein). Breeding to homozygosity (deletion of both alleles) will allow us to study the consequences of complete absence of

the individual protein. Eventually we will mate αA and αB knockout mice to produce mice totally devoid of α-crystallin.

2. We will complete sequencing of the αA-crystallin gene locus. Although much is known about regulation of the mouse αA-crystallin gene, the complete sequence of the gene has not yet been determined. Knowing the complete sequence of the gene and flanking regions will be beneficial in the location of possible regulatory sites not in the immediate 5′-flanking region of the gene. This knowledge will be invaluable in analysis of gene knockouts in stem cells and mice.

3. We will continue construction of vectors for use in transient transfection assays to locate additional regulatory elements in and around the αA-crystallin gene. This, along with the sequence of the locus, will help us to identify potential sites influencing the levels of gene expression.

NEI Research Program

Cataract—Lens Development and Aging

Laboratory of Ocular Therapeutics

Report of the Chief, Laboratory of Ocular Therapeutics

Peter F. Kador, Ph.D.

The Laboratory of Ocular Therapeutics (LOT) focuses on the development, evaluation, and mechanism of action of new ophthalmic drugs to treat eye diseases. The LOT research team is examining aldose reductase inhibitors (ARIs) and anticataract agents. In pursuing the development of more effective and less toxic ARIs, the efforts are progressing toward the development of an inhibitor unrelated to previous ARIs. A patent application has been made, and efforts are concentrated on further characterizing this inhibitor using biochemical, pharmacological, and computer molecular design techniques. Studies designed to elucidate the specific mechanism(s) by which aldose reductase initiates diabetic complications also are being conducted.

In studies utilizing galactose-fed dogs, LOT investigators have established that retinal changes associated with diabetic retinopathy progress to the proliferative stage. The dog represents the first animal model that demonstrates clinical and histological changes found in all stages of retinopathy. Studies are now focused on the development of proliferative retinopathy in long-term galactose-fed dogs. In addition, investigators are analyzing the specific role of aldose reductase in neuropathy, thyroid changes, and immune system responses. Models for the evaluations of lens changes by magnetic resonance imaging also are being developed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00003-20 LOT

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pharmacology of Ocular Complications

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Peter F. Kador	Ph.D.	Chief	LOT, NEI
Others:	William Greentree	D.V.M.	IRTA Fellow	LOT, NEI
	Jun Inoue	M.S., Pharm.	Special Volunteer	LOT, NEI
	Yong Lee	Ph.D.	Staff Fellow	LOT, NEI
	Martin Lizak	Ph.D.	Staff Fellow	LOT, NEI
	Anita Bartoszko-Malik	Ph.D.	Visiting Fellow	LOT, NEI
	Kazuhiko Mori	M.D., Ph.D.	Visiting Fellow	LOT, NEI
	Heike Neuenschwander	M.D.	Special Volunteer	LOT, NEI
	Libaniel Rodriguez	Ph.D.	Staff Fellow	LOT, NEI
	Matteo Schaffhauser	Ph.D.	Visiting Fellow	LOT, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Ocular Therapeutics

SECTION

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.75	6.75	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Events leading to the onset of various ocular complications are being investigated. Specific studies include the role of the enzymes aldose reductase and aldehyde reductase in the onset and progression of retinopathy, cataract, keratopathy, pupil function changes, and iris and ciliary process structure changes associated with diabetes and galactosemia. In addition, methods for either delaying or preventing the onset and progression of these complications through the pharmacological control of these enzymes are being developed.

Also being studied are events leading to the formation of several types of cataracts, as well as methods for controlling the onset of these cataracts through pharmacological intervention.

Project Description

Additional Personnel

Robert Balaban	Ph.D.	National Heart, Lung and Blood Institute
Duane Miller	Ph.D.	Ohio State University College of Pharmacy, Columbus, OH

Objectives

This project is designed to (1) gain insight into the mechanisms by which polyol-induced ocular diabetic complications and cataracts are formed and (2) develop methods for their regulation.

Methods

Diabetes can be induced experimentally in animals through the injection of streptozotocin. Diabetes-related complications linked to the sorbitol pathway also can be induced in animals such as rats and dogs by feeding them a galactose-enriched diet. Cataract formation and clinical retinal changes in experimental animals can be monitored through fundus photography. Biochemical studies used for the purification of enzymes include column chromatography, polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, chromatofocusing, and high-pressure liquid chromatography (HPLC). Polyol levels were determined by gas-liquid chromatography (GLC). Immunological analyses include the use of enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), Western blots, and immunohistochemical techniques employing the coupled antibody DAB-PAP technique. Computational methods for enzyme analysis, inhibitor structure-activity studies, and pupil-function changes require the use of the NIH PROPHET computer system and Charm and Quanta computer systems from Molecular Design.

Major Findings

Biochemical studies.—Studies on defining the inhibitor site of aldose reductase and aldehyde reductase are continuing with the evaluation of a number of Michael addition affinity-labeled aldose reductase inhibitors (ARIs). Analogs of the ARIs AL1576 and alrestatin possessing sterically diverse substituents, Michael addition adducts, and haloalkyl groups have been synthesized and evaluated *in vitro* for their ability to inhibit and irreversibly bind rat lens aldose reductase and rat kidney aldehyde reductase.

Inhibitory potency in general was reduced in a series of alrestatin analogs in which the carboxyl acid was condensed with sulfonamide groups; however, inhibition increased with 5-substitution with either nitro or Michael addition adducts. In the spirofluorenehydantoin series, substitution in the 2 position with similar open- or constrained-ring Michael addition adducts did not result in increased inhibition. The selective introduction of Michael addition adducts can result in increased inhibitory activity and irreversible binding of aldose reductase but not aldehyde reductase. Specific nucleophilic residues on the enzyme also are being identified by subjecting the alkylated enzyme to trypsin digestion and subsequent chemical ionization mass spectroscopy.

The pharmacophore requirements of the inhibitor site and the location of reactive nucleophilic and electrophilic sites are being refined through the use of molecular modeling. The theoretical tools utilized for this study include the AM1 quantum chemical method and the fitting methods of Quanta 3.3 conducted on an SGI Crimson computer. These results were then correlated to observed inhibitions of rat lens aldose reductase. Geometry optimization and energetics calculations have been conducted for a series of carboxyl acid-containing analogs of 9-spirofluorenehydantoin.

These calculations were conducted on the charged (-1) rather than neutral species because they are assumed to be charged at physiological pH. Superposition of these geometry-optimized compounds on AL1576 were conducted by utilizing both torsional flexible and rigid body fits, and the spatial regions at which reversible nucleophilic substitution takes place were then compared. The spirohydantoin AL1576 was chosen as a template in this study because (1) it contains a rigid hydantoin ring having two probable pharmacophores in which the relative position in space is fixed and (2) it has been shown to have high affinity for aldose reductase. These studies indicate that a negative charge center resides in the vicinity of the 2-oxygen atom of the hydantoin ring while the 4-carbonyl represents a region where a nucleophilic substitution likely takes place. Understanding the pharmacophore requirements will lead to the rational development of new ARIs. New, potent ARIs have been uncovered, and patent application has been made.

Recent studies have demonstrated that the dog is an excellent animal model for investigating ocular diabetic complications. Cataract development in the dog is similar to cataract development in humans with diabetes. The cataracts are characterized by formation of anterior and posterior superficial cortical opacities with the posterior polar region being more advanced. Furthermore, the dog develops advanced retinal changes similar to those clinically observed in human diabetics. Polyol formation initiated by the NADPH-dependent enzyme aldose reductase has been demonstrated to initiate these lens and retinal changes.

Magnetization transfer contrast (MTC) is a method in magnetic resonance imaging (MRI) that generates high-contrast images based on characteristic tissue differences resulting from the interaction of water and macromolecules. We are applying the MTC to document cataract formation and other structural changes of the anterior segment associated with diabetes eye complications in galactose-fed dogs. Dogs, sedated with acepromazine (i.m. injection), intubated, and then ventilated with 1.0-1.5% halothane are administered succinylcholine chloride to prevent eye movements and placed in a General Electric 2-T Omega MRI system.

M_s and M_o images are acquired using gradient-recalled echo sequences, with and without the saturation pulses, respectively, consisting of rf-irradiation 10 kHz off-resonance from the free-water proton signal. The T_{1sat} image data are obtained using one-short T_1-imaging, employing an inversion pulse followed by a series of small tip-angle pulses that sample the relaxation curve. These images are then compared with photographs obtained with photo-slit-lamp and retroillumination photography. The results indicate that MTC not only generates excellent images of the lens but also aids in the visualization of fine structures in the anterior segment, including the cornea, iris, ciliary bodies, choroid membrane, and Schlemm's canal.

^{19}F-NMR spectroscopy also is being used to measure *in vivo* aldose reductase activity in the dog lens by measuring the conversion of 3-deoxy-3-fluoroglucose to 3-deoxy-3-fluorosorbitol. This work is an extension of the *in vivo* evaluation of aldose reductase activity in rabbit lenses. Initial spatial coordinates for lenses are calculated from ^{1}H-images determined on a 2.0 Tesla GE Omega-CSI spectrometer. The SLOOP (spectral localization with optimal pointspread function) technique is then used with a proton decoupler to measure the accumulation of sorbitol in the rabbit lens. A double spin-echo sequence is utilized with selective excitation and refocusing pulses and with optimized phase-encoding gradient pulses using 1-sec repetition times and 25-msec echo times. SLOOP experiments indicate that 3-deoxy-3-fluorosorbitol can be observed in spectra of the anterior portion of the lens when adequate amounts of 3-deoxy-3-fluoroglucose are administered.

Retinal studies.—Vascular changes associated with diabetic retinopathy can be produced experimentally in beagles fed a 30% galactose diet. In studies designed to clarify the initiating lesions and progression of diabetic retinopathy, we have documented the progression of retinal lesions from background through the proliferative stage in the dog with ophthalmoscopic, fluorescein angiographic, and histopathologic findings. Initial retinal changes include aldose reductase-linked formation of pericyte ghosts and subsequent development of acellular capillaries, microaneurysms, and intraretinal hemorrhages. This early retinopathy progresses to include the appearance of occluded vessels, areas of nonperfusion, and intraretinal microvascular abnormalities (IRMA). Finally proliferative retinopathy develops, including the formation of fibrovascular membranes seen histologically on both the retinal surface and the posterior hyaloid membrane.

Pericyte ghost formation and the subsequent appearance of microaneurysms, intraretinal hemorrhages, and acellular capillaries associated with background retinopathy have been arrested in a dose-dependent manner in 36- to 38-month prevention studies utilizing 0.5, 5, 10, and 16 mg/kg/day of the ARI M79175. The dog represents the first animal model to demonstrate all the clinical and histological retinal vessel changes observed in human diabetics.

Significance to Biomedical Research and the Program of the Institute

Loss of vision from cataract and diabetic retinopathy are significant; therefore, methods for the pharmacological control of these ocular complications are required. We have developed an animal model that demonstrates advanced retinal vessel changes that are virtually identical both clinically and histologically to those observed in advanced diabetic retinopathy. Our present studies in dogs demonstrate for the first

time that loss of retinal pericytes, associated with aldose reductase, initiates retinal changes associated with both background and advanced diabetic retinopathy and that administration of ARIs in prevention studies can ameliorate the loss of pericytes and subsequent microaneurysms and retinal hemorrhages in a dose-dependent manner. The successful development of noninvasive methods for monitoring aldose reductase activity by nuclear magnetic resonance (NMR) procedures may have a direct impact on ongoing and planned clinical trials in which this procedure could serve as a quantitative indicator of drug efficacy. Cataract is one of the major causes of blindness in the developing world. In addition, loss of vision due to cataract is one of the major health problems of both people with diabetes and the aging population in the United States.

Proposed Course

These studies will be continued. Currently discovered ARIs will be evaluated and developed pharmacologically. The inhibitor site will be probed further through the use of affinity labels so that more potent and specific inhibitors may be developed. Studies will be continued on the mechanisms through which aldose reductase induces diabetic complications in various tissues.

NEI Research Program

Retinal Disease—Diabetic Retinopathy, Sickle Cell Retinopathy, and Other Vascular Abnormalities

Publications

Bartoszko-Malik A, Schaffhauser M, Ghany-Abdel Y, Miller DD, Kador PF: Evaluation of novel aldose reductase inhibitor site probes. *Invest Ophthalmol Vis Sci* 34(suppl):280, 1993.

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Smar MW, Ares J, Nakayama T, Itabe H, Kador PF, Miller DD: Selective irreversible inhibitors of aldose reductase. *J Med Chem* 35:1117-1120, 1992.

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standing diabetic retinopathy. *J Clin Invest,* in press.

Woel V, Kuszak JR, Takahashi Y, Wyman M, Kador PF: An ultrastructural analysis of posterior migrating lens epithelial cells in cataracts of galactose-fed dogs. *Invest Ophthalmol Vis Sci* 34(suppl):916, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00275-02 LOT

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of NADPH-Dependent Reductases in Ocular Complications

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Sanai Sato	M.D., Ph.D.	Visiting Scientist	LOT, NEI
Others:	Peter F. Kador	Ph.D.	Chief	LOT, NEI
	Shigeru Fukase	M.D.	Visiting Associate	LOT, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Ocular Therapeutics

SECTION

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.35	1.35	0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☒ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The increased influx of glucose into the sorbitol pathway in diabetes results in the accumulation of sugar alcohol sorbitol, which is linked to the pathogenesis of various diabetic complications such as retinopathy, neuropathy, and nephropathy. The role of aldose reductase and related enzymes in polyol formation and the subsequent onset of these complications are being investigated.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

In diabetes, excess glucose results in an increased influx of glucose into the polyol pathway. The accumulated sugar alcohol, sorbitol, has been linked to the onset of various diabetic complications such as cataract formation, retinopathy, neuropathy, and nephropathy. The development of potent aldose reductase inhibitors (ARIs) represents a new pharmaceutical approach to the treatment of diabetic complications. Understanding NADPH-dependent enzymes in target tissues is a required first step in the design of ARIs. This project is designed to investigate aldose reductase and its related enzymes in various tissues where diabetic changes occur.

Methods

Biochemical techniques include gel filtration, affinity chromatography, electrophoresis, immunoblotting, and isoelectric focusing on high-pressure liquid chromatography. Gas chromatography is used to identify and quantitate sugars. *In vitro* culture techniques include the culture of retinal capillary pericytes and endothelial cells, leukocytes, and fibroblasts. The results, including enzyme kinetic evaluations, are calculated using the NIH PROPHET computer system.

Major Findings

Human kidney.—Like that of the rat and dog, human kidney cortex contains predominantly aldehyde reductase rather than aldose reductase, whereas the medulla contains aldose reductase. The kinetic properties of aldose and aldehyde reductases purified from human kidney are essentially identical to those of the rat and dog kidney enzymes. Moreover, as demonstrated with rat kidney enzymes, aldehyde reductase—in addition to aldose reductase—generates polyols from both glucose and galactose in an *in vitro* incubation system. As in rat kidney, aldehyde reductase contributes to polyol formation in human kidney cortex, where diabetic changes occur. The use of animal models is based on the premise that similar pathological changes occur in both experimental animals and humans. Evidence that both human kidney aldose and aldehyde reductases are similar to the rat and dog enzymes in kinetic properties and inhibition by aldose reductase inhibitors gives enzymatic rationale for this approach.

Rat lens.—Rat lens displays dehydrogenase activity with naphthalene dihydrodiol as substrate. This dehydrogenase activity corresponds to aldose reductase throughout all purification procedures, which include gel filtration, affinity chromatography, and chromatofocusing. The dehydrogenase activity is observed with the highly purified aldose reductase and also with recombinant enzymes from the rat lens aldose reductase clone. The evidence indicates that, in rat lens, dehydrogenase activity in the presence of naphthalene dihydrodiol comes from aldose reductase.

Significance to Biomedical Research and the Program of the Institute

Despite the establishment of insulin therapy, the risk of loss of vision due to diabetic complications is still significantly high. Based on evidence that excess amounts of polyols are linked to the onset of diabetic complications, worldwide efforts have been made to develop ARIs. The full understanding of NADPH-dependent reductases and polyol formation will provide essential information on their clinical potency and contribute to further development of more potent inhibitors. Evidence that aldose reductase responds to naphthalene dihydrodiol with dehydrogenase activity may expose new facets of the role(s) of aldose reductase in certain types of toxic cataract.

Proposed Course

We will continue our evaluation of the location and enzymatic properties of aldose and aldehyde reductases in various tissues in which diabetic complications occur. To investigate retinal pericytes and endothelial cells as keys in diabetic retinopathy, we will utilize cell culture techniques. Leukocytes and various leukemia cells also will be investigated.

NEI Research Program

Diabetic Complications
Cataract Research

Publications

Fukase S, Mori K, Sato S, Kador PF: Comparison of NADPH-dependent reductases in dog lens and

leukocytes. *Invest Ophthalmol Vis Sci* 34(4) (suppl):757, 1993.

Sato S: Aldose reductase is the major protein associated with naphthalene dihydrodiol dehydrogenase activity in rat lens. *Invest Ophthalmol Vis Sci* 34:3172-3178, 1993.

Sato S, Kador PF: Human kidney aldose and aldehyde reductases. *J Diab Compl* 7:179-187, 1993.

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Laboratory of Retinal Cell and Molecular Biology

Report of the Chief, Laboratory of Retinal Cell and Molecular Biology

Gerald J. Chader, Ph.D.

The research focus of members of the Laboratory of Retinal Cell and Molecular Biology (LRCMB) is on elucidating new genes and biochemical mechanisms and learning the underlying causes of ocular diseases. In this way, we hope to intervene in the disease process before substantial damage to vision has been done or to apply rational methods of gene therapy before the terminal stages of the disease have been reached. Most of the approaches taken are molecular biological, molecular genetic, and/or candidate gene approaches.

The work of the Laboratory members is within the following National Institutes of Health strategic initiatives and National Eye Institute priorities: (1) molecular medicine, (2) gene research and gene therapy, and/or (3) research of high clinical relevance.

Within the LRCMB, the following three areas are emphasized:

1. Molecular biology and molecular genetics,
2. Gene therapy, and
3. Immunopathology.

Molecular Biology and Molecular Genetics

Specific advances made in the area of molecular biology and molecular genetics are discussed below.

Retina-specific genes.—Several genes that are predominantly or exclusively expressed in ocular tissues have been identified by subtractive cloning. Retina-specific genes and genes located on the short arm of the X-chromosome are pinpointed. These genes are being localized chromosomally to determine whether they are linked to eye diseases. Concurrently, genomic cloning and sequencing are being done to generate appropriate markers and polymorphisms. Cell lines are being immortalized in tissue culture such that subsequent laboratory experiments can be conducted.

Genes specific to retinal pigment epithelium (RPE).—Little is known about the specific complement of genes in the RPE or how these could be involved in diseases of the visual system. Thus, cloning of genes unique to RPE and its functioning is of importance. A new 65-kD protein of potential immunologic importance has been isolated from the human RPE. The gene has been cloned, allowing for study of tissue-specific expression. This gene is the first RPE-specific gene to be reported and characterized.

Photoreceptor-specific genes.—An effort has been made to identify and characterize genes for proteins and enzymes that are critical in functioning of the photoreceptor outer segment. For example, two proteins of the phototransduction cascade, S-antigen (S-Ag) and phosducin, have been well characterized as to their expression control. Both proteins are specific to the rod neuron and interact with visual cycle components (e.g., opsin). cDNAs and genomics for S-Ag and phosducin have been cloned and thoroughly analyzed, allowing for current advances in our understanding of expression, function, and pathology of the gene products.

Interphotoreceptor retinoid-binding protein (IRBP).—IRBP also is a critical link in the chain of enzymes and proteins that make up the visual cycle. Because of the huge size of the gene, however, it is very difficult to sequence in potential disease cases. We are thus cloning the homolog of the human IRBP gene from *Drosophila megalogaster* (i.e., fruitfly). Interestingly, it maps to an area of the *Drosophila* genome that is rich in mutants of ocular disease. Knowing the characteristics (e.g., erg) of the different diseases in the fly, we hope to pinpoint a specific human population with similar characteristics and examine the gene for defects in specific human families.

Pigment epithelium-derived factor (PEDF).—We have identified a novel neurotrophic protein, PEDF, synthesized by fetal human RPE cells that may be critical in the development of retinal photoreceptors. At very low concentration, PEDF causes the exten-

sion of elaborate neuronal processes from cultured retinoblastoma cells. Since these cells are thought to be derived from photoreceptor cone cells, we hope that PEDF can be as effective on cone neuron development *in vivo*. An important possibility is that, in the Royal College of Surgeons rat, a defect in the PEDF gene could cause retinal degeneration. Also, we are continuing evaluation of the clinical use of PEDF in retinal transplantation in collaboration with Dr. M. del Cerro. The molecular biology of this potentially very important neurotrophic agent is now being studied for application to retinal dysfunctions.

Fatty acid and tubulin defects in retinal degeneration.—In collaboration with Dr. Muriel Kaiser-Kupfer (Ophthalmic Genetics and Clinical Services Branch), we are investigating fatty acid uptake and metabolism in Bietti's crystalline retinopathy and a tubulin acetylation defect in a form of atypical retinitis pigmentosa (RP) for which we hope to elucidate the specific defects. Significant progress has been made in pinpointing the metabolic problems expressed in both these hereditary conditions.

Gene Therapy of Retinal Diseases

Points of focus and advances made within the area of gene therapy are discussed below.

Transgenic studies.—The IRBP and S-Ag genes are the best studied retinal genes other than rhodopsin. *Cis*-acting elements controlling the IRBP and S-Ag promoters, and thus their protein expression, have been identified in transfected human cells and in transgenic mice. Transgenic studies, in particular, have helped to uncover factors controlling gene activation in the embryonic period, specifically in the photoreceptor cell.

Gene analysis systems in transgenic mice and in transient transfections in cultured human retinoblastoma cells have been established for IRBP. Much of the 5′-flanking region of IRBP has been thoroughly examined to date. Similarly, a good deal of progress has been made with the S-Ag promoter. Enhancer elements necessary for expression are being defined through target mutagenesis studies. Tissue- and stage-specific elements, including TATA and CAAT boxes, are being defined as to retinal expression. This work is important in that specific molecules can be "gene-targeted" to the retina with precision.

Gene therapy.—Ribozymes are specifically constructed RNA species that can control expression of proteins within cells. By linking these simplified gene forms to appropriate promoters and utilizing a suitable transfer vector, we can construct new therapeutic modalities. Gene therapy can then be planned to treat autosomal dominant disorders that are currently unmanageable.

Ribozyme constructs for IRBP have been designed and are being studied in a transfected human retinoblastoma cell system. Concurrently, transgenic mice have been reared to determine if an RP-like condition is produced through down-regulating IRBP synthesis. Once perfected, ribozymes should be useful, not only with IRBP-related retinopathies but in conditions such as diabetic retinopathy and retinopathy of prematurity, disorders which probably involve overexpression of normal proteins such as growth factors.

Immunopathology

Most of this work is in collaboration with investigators in the Laboratory of Immunology. The focus is on the induction of experimental autoimmune uveitis.

Immunopathology.—Collaborative work with Dr. Igal Gery continues on the study of uveitis. The immunopathological site(s) of IRBP are being dissected in the Lewis rat and in the human with the final goal of controlling or preventing the disease process in man.

Immunogenetics.—Collaborative work with Dr. Rachel Caspi has established the IRBP-mouse model for experimental autoimmune uveoretinitis as very useful for studying the genetics of the disease and its relapsing characteristics.

Antigen presentation.—Collaborative work with Drs. Gery, Marc de Smet, and Robert Nussenblatt has demonstrated the presence of a 70-kD cell surface protein of B cells that specifically binds the major immunopathological determinant of IRBP. It is thought that this protein may function as a molecular chaperone in antigen presentation. It is a likely candidate for gene therapy in uveoretinitis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00070-16 LRCMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Vitamin A and Ocular Tissues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Barbara Wiggert	Ph.D.	Head, Section on Biochemistry	LRCMB, NEI
Others:	Kalpana Rengarajan	Ph.D.	Visiting Fellow	LRCMB, NEI
	R. Krishnan Kutty	Ph.D.	Senior Staff Fellow	LRCMB, NEI
	Todd Duncan	M.S.	Biologist	LRCMB, NEI
	Geetha Kutty	M.S.	Visiting Associate	LRCMB, NEI

COOPERATING UNITS *(if any)*

U. Lund, Sweden (T. van Veen, Ph.D.); U. Illinois Coll. of Med., Chicago (D. Pepperberg, Ph.D., T.-I. Okajima, Ph.D., H. Ripps, Ph.D.); Med. U.S.C. (R. Crouch, Ph.D., S. Hazard, Ph.D.); SLU Inst. F. Kir, Sweden (K. Narfstrom, D.V.M., Ph.D.); U. Hosp., Utrecht, The Netherlands (B. Zonnenberg, M.D., Ph.D.); U. Maryland Med. Sch. (M. Rodrigues, M.D., Ph.D.); Medical College of Georgia (S. Smith, Ph.D.); Emory Eye Center (J. Nickerson, Ph.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Biochemistry

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies on the physicochemical characteristics of a fatty acid-binding site on the interphotoreceptor retinoid-binding protein (IRBP) with fluorescent fatty acid analogs demonstrated that fatty acids were bound in a hydrophobic environment, that there was a single, specific fatty acid-binding site for each molecule of IRBP, and that there was nonradiative energy transfer from tryptophan residues to bound ligand. Probing the microenvironment of bound fluorophore with a quencher indicated a highly structured binding site.

Studies of the formation and release of 11-*cis* retinal by the retinal pigment epithelium at a physiological concentration of IRBP demonstrated that a sequential (i.e., unbranched) pathway mediates the processing of all-*trans* retinol to 11-*cis* retinal and its transfer to IRBP.

In the $mi^{vit}mi^{vit}$ mutant mouse model of retinal degeneration, retinyl palmitate was elevated fourfold and IRBP was elevated twofold in the eyes of affected mice, as compared with that in controls at 6-8 weeks of postnatal development. At the same time, IRBP mRNA was not elevated. The elevation in retinyl palmitate may be a significant factor in the retinal degeneration in this mutant, and IRBP turnover may be affected by an aberration in retinoid metabolism.

A 72-kDa heat shock protein (hsp) which bound specifically to peptide 1169-1191, a potent uveitogenic determinant of IRBP, has been identified in Lewis rat B cells and Epstein Barr virus-transformed B cells from normal human donors and uveitis patients. This hsp has a potential role in antigen processing and presentation by antigen-presenting cells.

Project Description

Additional Personnel

Igal Gery	Ph.D.	Head, Section on Experimental Immunology, LI, NEI
Rachel Caspi	Ph.D.	Visiting Associate, LI, NEI
Tatiana Putilina	Ph.D.	Visiting Associate, LRCMB, NEI
Mark de Smet	M.D.	Visiting Scientist, LI, NEI

Objectives

The purpose of this research project is to investigate the role of specific retinoid-binding proteins, such as interphotoreceptor retinoid-binding protein (IRBP), in mediating the action of retinoids in both normal and diseased ocular tissues.

Methods

Affinity chromatography, fluorescence spectroscopy, high-performance liquid chromatography, SDS-polyacrylamide gel electrophoresis, Western blotting, Northern blotting, slot-blotting, and the enzyme-linked immunosorbent assay were used to study retinoid-binding proteins.

Major Findings

The physicochemical characteristics of a fatty acid-binding site on IRBP were examined using a set of fluorescent fatty acid analogs with an anthracene moiety attached at different positions along the hydrocarbon chain. The results demonstrated that fatty acids were bound in a hydrophobic environment, as indicated by a blue shift in fluorescence maxima and by an increase in quantum yield of the bound ligand. A single, specific fatty acid-binding site existed for each molecule of IRBP with an apparent $K_d=3.6\times10^{-7}$M. There was nonradiative energy transfer from tryptophan residues to bound ligand, as well as fluorescence energy transfer to all-*trans* retinol when this ligand was bound to IRBP.

The interactions of IRBP and bound fatty acids were sensitive to denaturation by increasing concentrations of urea as judged by changes in nonradiative energy transfer efficiency and the quantum yield of the bound probe. Quantum yields of bound fatty acid analogs varied with position of the fluorophore along the hydrocarbon chain and had the lowest values for the fluorophore located at the midpoint. Probing the microenvironment of bound fluorophore with a quencher indicated a highly structured binding site.

In studies of the formation and release of 11-*cis* retinal by the retinal pigment epithelium (RPE) in the toad eyecup preparation, the time course of the specific activity of radio-labeled 11-*cis* retinal at a fixed, near-physiological IRBP concentration demonstrated that a sequential (i.e., unbranched) pathway mediates the processing of all-*trans* retinol to 11-*cis* retinal and its transfer to IRBP.

In the mutant mouse model of retinal degeneration, levels of retinyl palmitate in the eyes were elevated fourfold greater than controls by 8 weeks, and the levels remained elevated through 42 weeks. Levels of 11-*cis* retinal, all-*trans* retinol, and all-*trans* retinal were similar to those of controls, as were plasma retinol levels. Levels of IRBP at 2-4 weeks were similar in affected and control animals, but by 6 weeks IRBP levels were twofold greater in the affected mice than in controls and remained high for several weeks until degeneration of photoreceptor cells took place. At the same time, IRBP mRNA was not increased in the affected mice, indicating that IRBP turnover probably decreased in the disease. The elevation of retinyl palmitate may be a significant factor in the retinal degeneration seen in the mouse mutant, and IRBP turnover may be affected by an aberration in retinoid metabolism.

Peptide 1169-1191 is a potent uveitopathogenic determinant of IRBP. Recently a new class of proteins known as chaperones, which are part of the heat shock protein (hsp) family, have been implicated in antigen presentation and appear to prevent further degradation of antigen by lysosomes. A 72-kD protein that bound specifically to peptide 1169-1191 was found in Lewis rat B cells and EBV-transformed B cells from normal human donors and uveitis patients. This protein reacted with antibodies specific for both constituitively expressed and inducible 72/73-kD HSP 70 proteins and could have a potential role in antigen processing and presentation by antigen-presenting cells.

Large-scale purification of IRBP was continued for studies on the production of experimental autoimmune uveitis (EAU) in rats and mice and possible modes of suppression of the disease.

Significance to Biomedical Research and the Program of the Institute

Because of its importance in normal photoreceptor cell physiology (i.e., in facilitating the transport of retinoids during the visual cycle as well as the transport of fatty acids that are essential to normal function), abnormalities in IRBP function resulting from changes in concentration, distribution, or affinity for retinoids or fatty acids could be important either directly or indirectly in visual cell pathogenesis.

Proposed Course

Studies on the fatty acid- and retinoid-binding sites on IRBP will be continued to elucidate the relationship between the two ligands and the possible effect of fatty acid binding on the binding of retinoids and the function of IRBP in the interphotoreceptor matrix. We also will continue to study the physiological role of IRBP in the visual cycle, particularly the effect of removing IRBP during retinal detachment on regeneration of visual pigment. Continued studies on the $mi^{vit}mi^{vit}$ mouse model of retinal degeneration will include studies of retinoid metabolism in the RPE to determine the mechanism causing large elevations in retinyl palmitate in mutant mice. Further work also will be carried out to characterize the 72-kD hsp, which binds the uveitogenic peptide 1169-1191 of IRBP, and its possible role in human disease. We will continue to conduct large-scale purification of IRBP protein for studies of EAU.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

Hara Y, Caspi RR, Wiggert B, Chan CC, Streilin JW: Use of ACAID to suppress interphotoreceptor retinoid binding protein-induced experimental autoimmune uveitis. *Curr Eye Res* 11:97-100, 1992.

Kutty RK, Kutty G, Duncan T, Nickerson J, Chader GJ, Wiggert B: Radioanalytic estimation of amplification products generated by RT-PCR using (alpha-^{33}P) deoxynucleotide triphosphate. *Biotechniques*, 15:808, 811-812, 1993.

Pepperberg DR, Okajima TL, Wiggert B, Ripps H, Crouch RK, Chader GJ: Interphotoreceptor retinoid-binding protein (IRBP), in *Molecular Neurobiology*. Clifton, NJ, Humana Press Inc, 1993, in press.

Putilina T, Sittenfeld D, Chader GJ, Wiggert B: Study of a fatty acid binding site of interphotoreceptor retinoid-binding protein using fluorescent fatty acids. *Biochemistry* 32:3797-3803, 1993.

Rajagopalan S, Rodrigues MM, Wiggert B, Advani SH, Nair CN, Nickerson JM: Retinoblastoma: Interphotoreceptor retinoid-binding protein mRNA analysis by polymerase chain reaction. *Ophthalmic Paediatr Genet*, in press.

Sasamoto Y, Kawano YI, Bouligny R, Wiggert B, Chader GJ, Gery I: Immunomodulation of experimental autoimmune uveoretinitis by intravenous injection of uveitogenic peptides. *Invest Ophthalmol Vis Sci* 33:2641-2649, 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00196-10 LRCMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Genetics of the Eye and Ocular Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	Diane E. Borst	Ph.D.	Senior Staff Fellow	LRCMB, NEI
	Steven Bernstein	Ph.D., M.D.	Senior Staff Fellow	LRCMB, NEI

Others:

COOPERATING UNITS *(if any)*

Emory University, Atlanta, GA (J.M. Nickerson, Ph.D., J-S. Si, M.D.); University of Michigan, Ann Arbor (E. Farr, M.D.); University of Texas, Dallas (R. Hammer, Ph.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Gene Regulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects
☐ (a1) Minors
☐ (a2) Interviews

☒ (b) Human tissues

☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Interphotoreceptor retinoid-binding protein (IRBP) is an abundant glycolipoprotein that is expressed in the retina and pineal gland. IRBP mRNA is synthesized by the photoreceptor cells of the retina. We are characterizing the *cis*-elements regulating IRBP expression, using a transient transfection assay and transgenic mice. There are two conserved areas of sequence in the 5′-flanking regions of the bovine, human, and mouse IRBP genes, one from -1 to -350 and another at -1200 to -1410. The 5′-flanking region is necessary for expression of IRBP in transient transfection assays in Y79 retinoblastoma cell cultures. In transgenic mice, the same region also shows promoter activity in the retina and pineal, demonstrating that tissue specificity is engendered within the tested 5′-flanking regions of the gene.

Project Description

Additional Personnel

Eric Wawrousek	Ph.D.	Research Biologist, OSD, NEI

Objectives

This research is designed to (1) define the *cis*-acting elements and *trans*-acting factors that regulate interphotoreceptor retinoid-binding protein (IRBP) gene expression in a tissue and/or developmentally specific manner, (2) down-regulate eye-specific genes by exogenously derived genetic elements introduced either as antisense/catalytic RNA or antisense DNA oligonucleotides, and (3) use antisense technology in determining the pathophysiological role of aldose reductase in diabetic pathology. In addition to the obvious gene therapeutic possibilities, these approaches also can be used when total deletion of a target gene would be deleterious to the survival of the organism.

Methods

These studies use conventional techniques for cloning and analysis of nucleic acids. Transgenic mice have been made using standard techniques. Transgenic rats containing an antisense construct for aldose reductase are being produced in collaboration with Dr. Robert Hammer. Chloramphenicol acetyl transferase (CAT) activity is measured by an enzyme-linked immunosorbent assay (ELISA) or the biphase assay.

Catalytic RNA/antisense constructs (ribozymes) have been used for permanent transfection of cell lines that actively transcribe the messenger for IRBP. In addition, the transgenic animals produced express high levels of ribozymes targeted against endogenous IRBP mRNA. IRBP mRNA levels are measured quantitatively by techniques based on polymerase chain reaction (PCR), and by Northern analysis.

Major Findings

Gene expression.—Constructions containing presumptive elements of the IRBP promoter joined to an indicator gene (CAT) were made with both bovine and mouse IRBP promoters for study of the expression of the IRBP gene. Two sites in the 5′-flanking (promoter) region show significant homologies across species, and we have made constructions containing (1) both conserved blocks, (2) only one of the two blocks, or (3) neither blocks. These constructions were tested in several systems, including retinoblastoma cells (Y79 and WERI), frog oocytes, mixed pinealocyte primary cultures, transformed pinealocytes, and normal mouse fibroblasts. There is promoter activity in Y79 cells transfected with the IRBP promoter-CAT constructs containing both conserved blocks of sequence.

This is the first report of transfection of any retinoblastoma cell line yielding successful transient expression. In each block there is gel-shift experimental evidence for the binding of *trans*-acting factors confirmed in the proximal upstream area by DNAse footprinting experiments (collaboration with Drs. John Nickerson and Jing-Sheng Si). Southwestern blot analysis reveals a 120,000-MW protein that binds to the -300 region of the promoter. This binding activity is not unique to the retina, being present also in the heart, kidney, and lung; however, it is not ubiquitous.

DNA methylation.—DNA methylation is known to play a role in the regulation of gene expression. Experiments were done to determine the methylation state of the IRBP promoter in various tissues. DNA isolated from different tissues was digested with either Msp I or Hpa II and size-fractionated on agarose gels. Msp I and Hpa II are isoschizomers, but Msp I will digest the sequence when the 3′ cytosine is methylated, whereas Hpa II will not. Southern blots of mouse tail, liver, and retina DNA were probed with a labeled 1.6-kb piece of the mouse IRBP promoter. The autoradiographs show that the IRBP promoter is hypomethylated in the retina but not in the liver or the tail. This indicates that DNA methylation may somehow be involved in the tissue-specific regulation of IRBP gene expression.

Downregulation of gene expression.—The effect of site specificity and varying complement length on ribozyme activity *in vitro* has been studied using *in vitro* partial duplex transcription, cloned ribozyme templates, and substrate fragments. Ribozyme activity can be "tuned" *in vitro* by varying complement length. This tuning is unique and target site specific.

We have developed ribozymes, targeted against different sites in the IRBP mRNA, which have high *in vitro* activity. These ribosymes have been used to generate transgenic mice that express these ribozymes in ocular and other tissues. Preliminary data

show that there are apparently significant differences in the embryonic survival and tissue-specific expression of transgene and IRBP mRNA in these different constructs.

We are also using mouse retinoblastoma cell lines derived from mice transfected with SV-40 large T antigen. We have characterized these lines in terms of photoreceptor-specific gene expression; they also express high levels of IRBP mRNA.

Sequence analysis of the mouse IRBP genome.—Sequencing the genomic clones encoding the mouse IRBP gene has shown that the mouse IRBP gene is similar in the coding regions to both human and bovine genes. They differ, however, in that the mouse fourth exon contains a 3′-untranslated region that is intermediate in length (1.0 kb) between bovine (2.4 kb) and human (0.7 kb) orthologs. We are examining the sequence to determine alternative splice sites that may explain the unique appearance of two IRBP mRNA-size classes, as well as the difference between the forms of uveitis in rat and mouse species.

Significance to Biomedical Research and the Program of the Institute

Elucidation of the gene sequences of IRBP is fundamental to understanding normal retina development and function. Findings from the transgenic mice carrying the IRBP ribozyme construct will yield much useful information on the role of IRBP in development, as well as the function of the retina during relative IRBP deficiency.

Proposed Course

We have finished the major structural studies on the IRBP gene. With this foundation of information and battery of cloned genes, we have begun to study the regulation of IRBP gene expression. Related questions about the consequences of abnormal or absent IRBP function can be investigated in transgenic mice and *in vitro* systems.

Gene expression.—A deletion series of IRBP-promoter plasmids has been made, and preliminary experiments indicate that both the distal and proximal conserved sequences are important for expression of the IRBP gene in Y79 cells. However, fewer than 205 bases of the 5′-flanking region are needed for basal promoter activity in the Y79 cells. Some of these constructions have been injected into fertilized mouse eggs, and offspring are being examined for the expression of the constructions. We will compare the expression of these constructions in transgenic animals and Y79 cells under different culture conditions. Preliminary studies show that the transgene is active in development as early as embryonic Day 9, but high levels of expression coincide with the beginning of outer segment elongation. Steady state adult levels are not reached until about postnatal Day 30.

In future studies, we will examine in other transgenic mouse lines gene expression in both the retina and several other tissues during development. We will characterize the *cis*-acting DNA sequences that bind proteins in the promoter region by making alterations to these sequences. We plan to isolate the proteins that bind to these elements by screening retina cDNA expression libraries by the established Southwestern blotting procedure. Preliminary screenings have yielded two potential clones.

Downregulation of gene expression.—Mouse lines containing the IRBP ribozyme constructs are being analyzed concurrently with ocular histology and electrophysiological studies to assess the role of IRBP deficiency in ocular pathology. Transgenic rats expressing antisense for aldose reductase are currently being analyzed. A downregulation of aldose reductase in these animals should yield delayed galactose-induced cataractogenesis and resistance to diabetes-induced histopathology, confirming the importance of AR in these pathologic states.

NEI Research Program

Retinal Diseases—Photoreceptors and Retinal Pigment Epithelium

Publications

Humayun M, Bernstein SL, Gould HB, Chavis RM: Orbital childhood acute lymphoblastic leukemia as the initial presentation. *J Pediatr Ophthalmol Strabismus* 29:252-255, 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00124-13 LRCMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Metabolism of the Retina and Pigment Epithelium

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gerald J. Chader	Ph.D.	Chief	LRCMB, NEI
Others:	Robert Waldbillig	Ph.D.	Expert	LRCMB, NEI
	Bruce Pfeffer	Ph.D.	Senior Staff Fellow	LRCMB, NEI
	Joyce Tombran-Tink	Ph.D.	Staff Fellow	LRCMB, NEI
	Stephen Gaudet	Ph.D.	Staff Fellow	LRCMB, NEI
	S. Patricia Becerra	Ph.D.	Visiting Scientist	LRCMB, NEI
	Timothy Schoen	B.S.	Biologist	LRCMB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Gene Regulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.5	5.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies are focused on an understanding of the molecular biology and molecular genetics of the retina and hereditary retinal degenerations. The retina and pigment epithelium are neuroepithelial tissues that work in close cooperation. Specific growth and differentiating factors found in the eye guide development and interactions of individual ocular tissues to form a functional visual system. For example, ocular tissues synthesize a number of growth factors. There now appear to be several systems that could self-regulate growth and metabolic activity in the retinal pigment epithelium and that could be involved in eye diseases. In this regard, we have cloned and characterized a unique differentiating protein secreted from fetal human pigment epithelial cells, called pigment-epithelium-derived factor, that is neurotrophic to cultured human retinoblastoma cells and may affect neural retinal development *in vivo*. This protein maps to chromosome 17p, where there is a cluster of cancer-related genes. It is a prime candidate in the hereditary retinal dystrophy observed in the Royal College of Surgeons rat.

Project Description

Objectives

Our objective is to obtain a better understanding of the molecular biology and molecular genetics of ocular tissues in health and disease. Study of growth and differentiation factors, be they protein (e.g., pigment epithelium-derived factor [PEDF]) or polypeptide (e.g., insulin-like growth factor [IGF]-1), is critical in obtaining a view of the events that control the early development of the eye and in maintaining normal function in the adult.

Methods

Molecular biological, genetic, and immunocytochemical techniques are used. Tissue culture is used to grow cells. In particular, the human retinoblastoma cell line, Y79, is used as a test system for differentiating agents.

Major Findings

Hereditary diseases often occur in the presence of genes important in cell division and differentiation. PEDF seems to be such a gene product. It is secreted by cultured fetal human pigment epithelial cells and appears to be present in the normal adult interphotoreceptor matrix. The protein migrates at approximately 54 kD on SDS-polyacrylamide gels. PEDF causes marked differentiation of human Y79 retinoblastoma cells in culture. This differentiation is characterized by an extensive elongation of neurite-like processes and a gathering of cells into "rosette-like" aggregates. Immunocytochemistry shows that the expression of specific neuronal markers also is enhanced. Thus, PEDF is a unique protein, synthesized and secreted by retinal pigment epithelial cells, that could direct early development, even early in embryogenesis. It may be that PEDF also is present after the important developmental period and may help to maintain retinal cell viability in the adult retina.

We have cloned the cDNA for the PEDF gene, and have determined that the protein is a member of the SERPIN (serine protease inhibitor) superfamily of genes. Some members of this family are known to promote cellular differentiation, making it more probable that PEDF has a major, similar role in the retina. Using fluorescent *in situ* hybridization, polymerase chain reaction, and Southern blotting, we have localized the PEDF gene to the short arm of human chromosome 17. Through analysis of somatic cell hybrids containing only specific regions of 17p and 17q, we have further pinpointed PEDF to 17p13-.1. It is important that PEDF colocalizes to the chromosomal area that contains the Li-Fraumeni cancer gene and a number of yet unknown cancer genes. Thus, PEDF may be part of an important cluster of genes involved in cellular proliferation and cancer as well as a prime candidate gene in the retinal dystrophy in the Royal College of Surgeons rat. The recombinant protein, which has now been expressed in *Escherichia coli* cells, has been shown to be an active neurotrophic agent. The availability of relatively large amounts of recombinant PEDF should allow for more direct studies on its role(s) in ocular development and disease.

In parallel work, we have evidence implicating IGF-1 in visual development. In the eye, IGF-1 seems to participate in the attainment of overall size of the eye and in the function of individual ocular tissues and cell types. Specific IGF-binding proteins (IGFBPs) are known to control the bioavailability of the IGFs and thus are important regulators of IGF activity in health and disease. The vitreous and several ocular tissues contain very high levels of IGFBPs that are not derived from extraocular sources. The ciliary body is the probable source of synthesis of at least one of the vitreal binding proteins (BPs), specifically IGFBP2. We believe that the ciliary body probably secretes the BP into the vitreous, where it could be a major factor in regulating developmental programs in the eye. Interestingly, the cornea exhibits exceptionally high amounts of IGFBP activity. Its role in corneal metabolism is yet unknown but, because of their growth-regulating potential, BPs could be involved in important processes such as wound healing and corneal complications of diabetes.

Significance to Biomedical Research and the Program of the Institute

Determining the genes that control normal ocular growth, differentiation, and function and studying them on molecular biological and molecular genetic levels will aid us in understanding eye diseases, especially those of a hereditary, early developmental nature. With such knowledge, we can apply rational methods of gene therapy to ocular diseases.

Proposed Course

The molecular biology and molecular genetics of ocular development will be further examined. We will investigate the factors that affect normal and abnormal growth. Examination and analysis of the full PEDF gene will help to elucidate its presumptive role(s) in retinal development. The recombinant PEDF protein will be used to elucidate the role of the novel new protein in retinal disease processes.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

Becerra SP, Palmer I, Kumar A, Steele F, Shiloach J, Notario V, Chader GJ: Overexpression of fetal human pigment epithelium-derived factor in Escherichia coli: A functionally active neurotrophic factor. *J Biol Chem*, 268:23148-23156, 1993.

Boje KM, Skolnick P, Raber J, Fletcher RT, Chader GJ: Strychrine-insensitive glycine receptors in embryonic chick retin: characteristics and modulation of NMDA neurotoxicity. *Neurochem Int* 20:473-486, 1992.

Gaudet SJ, Chader GJ: Partial purification and characterization of arylamine-N-acetyltransferase in bovine retina. *Curr Eye Res* 11:1185-1192, 1992.

Gaudet SJ, Hayden BJ, Chader GJ, Namboodiri MA: Differential regulation of arylamine and arylalkylamine N-acetyltransferases in human retinoblastoma (Y-79) cells. *Neurochem Int* 22:271-275, 1993.

Schoen TJ, Beebe DC, Clemmons DR, Chader GJ, Waldbillig RJ: Local synthesis and developmental regulation of avian vitreal insulin-like growth factor-binding proteins: A model for independent regulation in extravascular and vascular compartments. *Endocrinology* 131:2846-2854, 1992.

Steele FR, Chader GJ, Johnson LV, Tombran-Tink J: Pigment epithelium-derived factor (PEDF): Neurotrophic activity and identification as a unique member of the serine protease inhibitor (SERPIN) gene family. *Proc Natl Acad Sci USA* 90:1526-1530, 1993.

Tombran-Tink J, Li A, Johnson MA, Johnson LV, Chader GJ: Neurotrophic activity of interphotoreceptor matrix on human Y79 retinoblastoma cells. *J Comp Neurol* 317:175-186, 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00148-20 LRCMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visual Control Mechanisms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gerald J. Chader	Ph.D.	Chief	LRCMB, NEI
Others:	Paul Wong	Ph.D.	Visiting Fellow	LRCMB, NEI
	Tatiana Putilina	Ph.D.	Visiting Associate	LRCMB, NEI
	Ignacio Rodriguez	Ph.D.	Staff Fellow	LRCMB, NEI
	Jun Li	M.D.	Visiting Associate	LRCMB, NEI
	Susan Gentleman	Ph.D.	Biologist	LRCMB, NEI
	R. Theodore Fletcher	M.S.	Chemist	LRCMB, NEI

COOPERATING UNITS *(if any)*

School of Veterinary Medicine, University of Pennsylvania (G. Aguirre, D.V.M., Ph.D.); Department of Anatomy, Erasmus University, Rotterdam, The Netherlands (S. Sanyal, Ph.D.); Department of Zoology, University of Lund, Lund, Sweden (T. van Veen, Ph.D.); Instituto Nazionale per la Ricera sul Cancro, Genova, Italy (A. Albini, Ph.D., D. Noonan, Ph.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Gene Regulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.5	5.5	1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☒ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Since gene therapy of the retina depends on knowledge of normal complements of tissue-specific genes and how they change in disease, we are studying the expression of specific gene products related to several hereditary diseases. If normal mechanisms fail, hereditary diseases of the retina such as retinoblastoma or retinitis pigmentosa will result. We have developed new techniques to clone and sequence retina-specific genes at a higher efficiency. We also have found that laminin, an important extracellular matrix protein, and cAMP, a small intracellular messenger, slow retinoblastoma cell growth and promote differentiation. Specifically, they switch development from a photoreceptor-like pathway to a conventional neuronal-like pathway. Progress also has been made in identifying apoptosis as a primary and unifying mechanism for cell death in several hereditary retinal degenerations. All these factors and processes could lead to more efficient gene therapy of the diseased neural retina.

Project Description

Objectives

Normal expression of genes in the retinal photoreceptor neuron is crucial to visual function in the adult. Thus, the factors that code for normal gene control and expression in human retina and in animal models of retinal degeneration are of primary interest. We also have mounted a major effort to develop new molecular biological techniques such that unique retinal and retinal pigment epithelial genes can be identified, cloned, and sequenced for ultimate use in screening human populations with inherited diseases of the visual system.

Methods

Standard molecular biological, biochemical, and neurochemical techniques are employed. Histochemical techniques are used when necessary.

Major Findings

1. Laminin is a ubiquitous extracellular matrix protein that has profound effects on a variety of cell types. For example, both gene and protein expression in cultured human Y79 retinoblastoma cells are switched from a photoreceptor to a conventional neuronal pathway by addition of this basement membrane glycoprotein in culture. Unlike other cell systems where laminin influences differentiation, Y79 cells cannot attach to or chemotactically respond to laminin. Cyclic AMP (cAMP) is also an intracellular messenger that can influence differentiation in several cell types. Using cultured human retinoblastoma cells as a model system, we have found both laminin and cAMP to have major positive influences on photoreceptor differentiation.

2. We are interested in developing new molecular biological techniques that will allow for more efficient identification of highly expressed genes of the retina-pigment epithelium complex. Each tissue of the body expresses a unique complement of genes that are transcribed and translated at a high level. In the retina and pigment epithelium, several very specific proteins are highly expressed, such that photoreception and the visual process can take place. Similarly, it is often a genetic defect in these tissue-specific genes that results in a hereditary degeneration such as retinitis pigmentosa. We have developed and are using new methods for rapid polymerase chain reaction-based construction of specifically enriched libraries from very small retinal samples. This is especially important because tissue samples are limited for studying early development and rare pathology samples. An important methodological advance involves subtractive cloning on an immobilizing base. We are now applying these techniques to the study of apoptosis (i.e., programmed cell death) in the retina and to the elucidation of fatty acid-binding proteins in normal and degenerating retinas. In apoptosis, in particular, it now appears that programmed cell death may be a common mechanism by which many hereditary defects initiate photoreceptor cell death.

Significance of Biomedical Research and the Program of the Institute

To control a hereditary disease process in a tissue and to reverse it through gene therapy, one must identify the normal complement of unique genes expressed in that tissue. This is especially true in an early degenerative process (e.g., retinitis pigmentosa) and in other hereditary diseases, such as retinoblastoma, in which abnormal changes are subtle and can be masked by normal developmental switches in gene expression. Thus, studying apoptosis and similar processes in the retina will lead to better methods for gene therapy in the neural retina.

Proposed Course

Molecular biological and developmental control mechanisms in the retina and pigment epithelium will continue. In particular, we will investigate gene expression in normal retinas and in retinas affected with specific genetic diseases. Apoptosis will continue to be a focus, since future gene therapy in retinal degenerations may depend on understanding how to prevent death of the photoreceptor neuron.

NEI Research Program

Retinal Diseases—Retinitis Pigmentosa and Other Inherited Disorders

Publications

Albini A, Melchiori A, Garofalo A, Noonan DM, Basolo F, Taraboletti G, Chader GJ, Gavazzi R: Matrigel promotes retinoblastoma cell growth in vitro and in vivo. *Int J Cancer* 52:234-240, 1992.

Hooks JJ, Robbins S, Wiggert B, Chader G, Detrick B: Can viruses trigger retinal degenerative processes? in Hollyfield JG, Anderson RE, LaVail MM (eds): *Progress in Clinical Biological Research, Degenerative Retinal Disorders: Clinical and Laboratory Investigations*, in press.

Kutty G, Duncan T, Nickerson J, Si JS, vanVeen T, Chader GJ, Wiggert B: Light deprivation profoundly affects gene expression of interphotoreceptor retinoid-binding protein in the developing mouse eye. *Exp Eye Res*, in press.

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Putilina T, Smith S, Gentleman S, Chader GJ: Rapid PCR-based construction of specifically enriched libraries from small retina samples. *Exp Eye Res* 54:825-826, 1992.

Wong P, Putilina T, Chader GJ, Tenniswood M: The human gene encoding TRPM-2 exists as a single gene locus on the short arm of chromosome 8. *Am J Human Genet*, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00260-04 LRCMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Outer Retina-Specific Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Michael Redmond	Ph.D.	Research Biologist	LRCMB, NEI
Others:	Suyan Liu	M.D., Ph.D.	Visiting Fellow	LRCMB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Gene Regulation

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☒ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Retinal pigment epithelium (RPE) cells and photoreceptor cells are functionally and developmentally closely integrated. Derangements of the RPE are involved in certain retinal diseases. However, the RPE is poorly understood at the molecular level. We are characterizing RPE65, a developmentally regulated, conserved 65-kD RPE-specific microsomal membrane-associated protein. We have cloned the cDNA for RPE65 and found that it encodes a novel protein. This protein does not have any predicted transmembrane segments, yet it has a strong affinity for phospholipids, which may be related to its function. The cDNA sequence is being used to overexpress RPE65 protein for functional studies. The potential role of the protein in inducing uveitis also will be studied using recombinant protein.

The lack of translation of RPE65 mRNA in cultured RPE cells is being investigated as a possible mechanism of posttranscriptional regulation that may have a bearing on the RPE-retina relationship as well as on RPE transplantation studies. We have isolated a full-length genomic clone for RPE65. It is at least 22 kb in length. We have used the cDNA and genomic sequences to localize the human gene for RPE65 to chromosome 1p31 and the mouse homolog to distal chromosome 3. These do not correspond to any ocular disease gene localized so far. Nonetheless, RPE65 remains a candidate gene for RPE-involved disease.

Project Description

Objectives

The retinal pigment epithelium (RPE) and the photoreceptor cell layer of the neural retina form a functionally and developmentally interdependent complex. Dysfunction of the RPE, accordingly, is deleterious to the photoreceptors and, hence, to vision itself. Despite these important considerations, little is known about the RPE at the molecular level. In this laboratory, we are cloning proteins specifically or preferentially expressed in the RPE with the aim of understanding mechanisms important to the RPE. Our major emphasis is on a 65-kD protein that we have named RPE65. We also are studying other RPE-expressed proteins.

Methods

Molecular cloning and biochemical and protein chemistry techniques are employed in this study. In addition, we are performing automated fluorescent DNA sequencing and gene mapping.

Major Findings

1. RPE65 is a developmentally regulated, membrane-associated, nonglycosylated 65-kD protein restricted to and conserved in vertebrate RPE. It is the major protein of the RPE microsomal fraction. This protein displays a calcium-independent affinity for phospholipids.

2. We have cloned a composite 3,115-bp cDNA for this protein and have shown it to encode a novel protein of 533 aa that matches exactly the authentic protein sequences from peptide fragments of RPE65. Recombinant protein expressed in *Escherichia coli* has the same molecular weight as native RPE65 and is recognized by the RPE9 monoclonal antibody.

3. mRNA for the protein, which is restricted to RPE, is abundant in primary cultures of RPE. However, these cultures do not express the protein, suggesting that the message is posttranscriptionally regulated *in vitro*.

4. We have isolated a human genomic clone for RPE65. It is at least 22 kb in length. We are now mapping and sequencing it.

5. We have localized the gene for the RPE65 to human chromosome 1p31 and to the far distal end of mouse chromosome 3. Neither of these loci matches that of a known ocular disease or phenotype.

Significance to Biomedical Research and the Program of the Institute

The RPE is poorly characterized at the molecular level, despite its pivotal role in the maintenance of photoreceptor function and, hence, in vision itself. We have identified RPE65 as a conserved, RPE-specific molecule that is developmentally expressed. cDNA sequencing demonstrates that it is a novel protein. The function of this protein, while not yet clear, may be related to its affinity for phospholipids. Elucidation of the basis for its posttranscriptional regulation *in vitro* may have significant bearing on the culture of RPE cells. This has some clinical significance because RPE cell transplantation is receiving much attention as a possible mode of intervention in treating some retinal diseases. In addition, because of its RPE specificity, the RPE65 gene can be considered a potential candidate gene for retinal disease. At present, however, neither its human nor its mouse chromosomal locations match those of any mapped disease loci. As more disease loci are matched, this may change. Again, in view of its RPE-specific expression, elucidation of its gene structure may uncover RPE-specific regulatory elements. Finally, in view of the involvement of the RPE in uveitis, it is possible that RPE65 is uveitogenic. Now that we have cloned the cDNA, it will be possible to overexpress the protein to test this hypothesis.

Proposed Course

1. The basis for the posttranscriptional regulation of RPE65 will be investigated.

2. The structure of the human RPE65 gene will be studied. The gene will be sequenced, and its regulatory regions will be analyzed. The mouse gene RPE65 will be compared with that of the human.

3. RPE65 will be tested as a possible RPE autoantigen. RPE65 protein will be overexpressed for this purpose.

4. Elucidation of the structure and function of RPE65 will continue. This will involve use of a variety of approaches.

5. Other RPE proteins will be cloned.

NEI Research Program

Retinal Diseases—Photoreceptors and Retinal Pigment Epithelium

Publications

Hamel CP, Tsilou E, Harris E, Pfeffer BA, Hooks JJ, Detrick B, Redmond TM: A developmentally regulated microsomal protein specific for the pigment epithelium of the vertebrate retina. *J Neurosci Res* 34:414-425, 1993.

Hamel CP, Tsilou E, Pfeffer BA, Hooks JJ, Detrick B, Redmond TM: Molecular cloning and expression of RPE65, a novel retinal pigment epithelium-specific microsomal protein that is post-transcriptionally regulated in vitro. *J Biol Chem* 268:15751-15757, 1993.

Redmond TM, Tsilou E, Pfeffer BA, Detrick B, Hooks JJ, Hamel CP: Cloning and expression of a novel retinal pigment epithelium-specific 65 kDa microsomal protein. *Invest Ophthalmol Vis Sci* 34(suppl):982, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00132-12 LRCMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Phototransduction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Toshimichi Shinohara	Ph.D.	Head, Section on Molecular Biology	LRCMB, NEI
Others:	Takanobu Kikuchi	Ph.D.	Visiting Associate	LRCMB, NEI

COOPERATING UNITS *(if any)*

Mount Sinai Hospital, Toronto, Canada (Martin Breitman, Ph.D.); Department of Anatomy, Nagoya University School of Medicine, Tsurumai, Showa-Ku, Nagoya, Japan (J. Usukura, M.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Molecular Biology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have characterized the S-antigen genes from human and mouse and the 33-K protein genes from mouse and human. The S-antigen genes were approximately 50 kbp in length, contained 16 exons and 15 introns, and comprised 97% intron and 3% exon. The 5′-flanking regions of the genes, approximately 1.5 kbp long, had no known regulatory elements for transcription, such as TATA, GC, or CCAAT boxes.

Regulatory sequences and nuclear factors governing tissue-restricted expression of the mouse arrestin gene were investigated. The results showed that, while proximal promoter sequence -38 to +304 are sufficient to direct low levels of retina-specific gene expression, sequences extending upstream to position -209 support higher levels of expression in the retina, as well as detectable expression in the lens, pineal gland, and brain. Within the interval between positions -205 and -185, a region which contains two direct repeats of the hexamer, TGACCT, the proximal promoter binds three apparently retina-specific nuclear factors—Bp1, Bp2, and Bp3—through overlapping sequences centered between positions -25 and -15. Bp1 and Bp3 also recognize a closely related sequence found in the promoter regions of several other vertebrate photoreceptor-specific genes. Moreover, the consensus binding site for Bp1, designated PCE1, is identical to RCS1, an element known to play a critical role in eliciting photoreceptor-specific gene expression in *Drosophila melanogaster*. The results suggest that PCE1 and RCS1 are functionally, as well as structurally, similar and that, despite marked differences in the fly and vertebrate visual system, the transcriptional machinery involved in photoreceptor-specific gene expression has been strongly conserved evolutionarily.

Project Description

Objectives

The objectives of this project are (1) to understand the basic mechanism of phototransduction in the retina and (2) to understand the structure, function, and evolution of the proteins present in photoreceptor rod cells and pinealocytes.

Methods

Conventional methods for analysis of proteins and nucleic acids being used include protein purification and RNA and DNA isolation, characterization, and sequence determination. Various recombinant DNA techniques also being used include a Baculovirus expression vector system, synthesis of point mutation clones, characterization of promoters, and transgenic animals. We also have synthesized and used purified oligopeptides and oligonucleotides.

Major Findings

1. The gene sequences of S-antigen (S-Ag) from human and mouse were determined. It is 50 kbp in length and has 15 introns and 16 exons. The smallest exon encodes for three amino acids.

2. The intron-exon map sequence of the mouse S-Ag gene has been well conserved. Approximately 97% of the S-Ag gene is intron and 3% is exon.

3. The human and mouse S-Ag cDNAs have been subcloned into two expression vectors and have been expressed. The products of S-Ag cDNA were purified by column chromatography and prepared for crystallization.

4. The 5′-flanking sequence of the human and mouse S-Ag genes were determined. Promoter activity was demonstrated in the *in vivo* and *in vitro* transcriptional assays.

5. Although the S-Ag promoter sequences are highly conserved between human and mouse, promoter activity was found at different locations of the 5′-flanking region in the human and mouse genes. This result suggests that the promoter activity is highly specific to tissues and species.

6. The mouse S-Ag promoter, 1,300 bp in length, was fused with the chloroamphenicol acetyltransferase (CAT) gene, and that gene was introduced into transgenic mice. The transgenic animals expressed CAT activity only in the retina and pineal gland. This result indicates that the promoters have a tissue-specific enhancer and promoter activity.

7. The opsin promoter was fused with a diphtheria toxin gene, and that fusion gene was introduced into transgenic mice, which subsequently lost only the photoreceptor rod cell layer.

8. Several cDNAs of Shuzin, a retinal photoreceptor protein, were isolated from human and cow retinal cDNA libraries (λ-gt11), and the entire DNA sequences were determined. The deduced protein has sequence similarity with TFIID. Its gene also was isolated from a genomic library and its DNA sequence was determined. It is composed of two introns and three exons.

9. Two genes of 33-kD ROS-specific proteins have been isolated from the retinal libraries of human and mouse, and the entire DNA sequence of these genes have been determined. They have four exons and three introns.

10. The proximal promoter sequence positions −38 to +304 are sufficient to direct low levels of retina-specific gene expression.

11. The proximal promoter binds three retinal specific nuclear factors (Bp1, Bp2, and Bp3) through overlapping sequences centered between positions −25 and −15.

12. The distal promoter sequence positions −205 to −185, a region which contains two direct repeats of the hexamer, TGACCT.

13. We found a consensus retinal photoreceptor-specific site (PCE1).

14. The transcriptional machinery involved in photoreceptor-specific gene expression has been strongly evolutionarily conserved.

Significance to Biomedical Research and the Program of the Institute

Eyes have remarkable properties in functioning efficiently over a wide range of illuminations. Rod cells, having photosensitive rhodopsin, are more sensitive to dim light; they adapt in the dark to increase their sensitivity. However, rod cells cease their sensitive phototransduction in bright light. In contrast, cone cells do not operate in dim light but are operative in bright light. Rhodopsin, transducin, phosphodiesterase, rhodopsin kinase, and S-Ag have been known to be associated with the phototransduction cascade. Rhodopsin kinase and S-Ag are

considered to be the important proteins for light-dependent modulation of phototransduction. To understand this light-dependent modulatory mechanism in rod outer segments, we have characterized S-Ag, Shuzin, and 33K protein as well as their genes. Interestingly, other signal transduction systems have cascades similar to that of phototransduction (one of the best characterized receptor-mediated signal transduction processes). In the phototransduction cascade, the shutoff mechanism appears to be modulated by the phosphorylation and dephosphorylation of rhodopsin. Studying this modulation mechanism is important for understanding phototransduction as well as for understanding signal transduction in general. In addition, we think that the night blindness of vision may in part be associated with light adaptation.

Proposed Course

The following studies are in progress or have been proposed for Fiscal Year 1993:

1. Identification of the S-Ag promoter using transgenic animals.

2. Identification of *cis*-acting factors of the S-Ag and 33K protein promoter.

3. The knockout of genes of S-Ag and phosducin. Investigation of a functional role for S-Ag and 33K protein, the homologous recombination between a mutant gene and a normal gene will be induced in ES cell culture. The recombinant ES cells will be introduced into a transgenic animal system in order to produce a mutant mouse.

NEI Research Program

Retinal Diseases—Photoreceptors and Retinal Pigment Epithelium

Publications

Ahe T, Kikuchi T, Chang T, Shinohara T: The sequence of the mouse phosducin gene and its 5'-flanking region. *Gene*, 133:179-186, 1993.

Danciger M, Kozak CA, Abe T, Shinohara T, Farber DB: The gene for retinal rod 33-kDa protein is on mouse chromosome 2, near lamb2. *Cytogenet Cell Genet*, 56:202-205, 1991.

Kikuchi T, Raju K, Breitman ML, Shinohara T: The proximal promoter of the mouse arrestin gene directs gene expression in photoreceptor cells and contains an evolutionarily conserved retinal factor-binding site. *Mol Cell Biol* 13:4400-4408, 1993.

Shinohara T, Kikuchi T, Tsuda M, Yamaki K: A family of retinal S-antigen (arrestin) and their genes: Comparative analyses of human, mouse, rat, bovine, and *Drosophila*. *Comp Biochem Physiol*, 103:505-509, 1992.

Usukura J, Khoo W, Abe T, Shinohara T, Breitman ML: Abnormal development of cone cells in transgenic mice ablated of cone rod photoreceptor cells. *Ann N Y Acad Sci*, in press.

Usukura J, Khoo W, Abe T, Shinohara T, Breitman M: Cone cells fail to develop normally in transgenic mice ablated of rod photoreceptor cells. *Tissue Cell*, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00250-06 LRCMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Experimental Autoimmune Uveitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Toshimichi Shinohara	Ph.D.	Head, Section on Molecular Biology	LRCMB, NEI
Others:	Dhirendra Singh	Ph.D.	Visiting Associate	LRCMB, NEI
	Siradanahalli Guru	Ph.D.	Visiting Fellow	LRCMB, NEI
	Shirley Yu	B.S.	Biologist	LRCMB, NEI

COOPERATING UNITS *(if any)*

Department of Ophthalmology, Miami University, Miami, FL (D. Hamasaki, Ph.D.); Department of Anatomy, Nagoya University School of Medicine, Tsuramai, Showa-ku, Nagoya, Japan (Jiro Usukura, M.D.)

LAB/BRANCH

Laboratory of Retinal Cell and Molecular Biology

SECTION

Section on Molecular Biology

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We had previously determined amino acid sequences of human, mouse, rat, and bovine retinal S-antigen (S-Ag) and rat pineal gland S-Ag. Immunogenic sites and four uveitopathogenic sites of S-Ag also were determined; two immunogenic sequences were highly conserved among the species. Many proteins in the National Biomedical Research Foundation data base have a sequence similar to that of a uveitopathogenic site. We chemically synthesized many peptides, some of which induced experimental autoimmune uveitis (EAU) and experimental autoimmune pinealitis (EAP) in Lewis rats. In addition, we found native yeast histone H3 capable of inducing EAU.

To understand the role in autoimmunity of infectious microorganisms which have cross-reactive antigens, we injected Lewis rats with peptide M, together with one of six different killed bacteria, with or without incomplete Freund's adjuvant (IFA). The rats injected with IFA developed EAU. To assess the impact of infection by live microorganisms, we injected low doses of live *Escherichia coli* expressing S-Ag and baker's yeast with a cross-reactive antigen into the rats several times. The rats injected with either live *E. coli* or live yeast developed EAU. We conclude that infection by microorganisms which have cross-reactive antigens can break immune tolerance to self-antigens and induce inflammatory autoimmune diseases.

As an extension of our previous EAU research, we speculated that some types of cataracts may be induced by autoimmune insults. To investigate this issue, we conducted similar experiments: Three groups of four rats were injected three times with lens homogenate, β-crystallins, or a β-crystallin (β-A1) emulsified with complete Freund's adjuvant (CFA). All the animals developed severe damage in lens epithelial cells 5 weeks from the date of the first injection. The rats injected with a synthetic peptide derived from *Salmonella typhimurium* protein, which has five amino acid residues identical to rat β-crystallin (β-B2), also induced similar damage. Infection by microbes having antigens homologous to the lens antigens can induce high levels of autoantibodies that provoke lens epithelial cell damage. Thus, autoimmune insult in lens epithelial cells may be an etiology of an initial stage of cataractogenesis. Our future research will focus more on autoimmunity in lens cataractogenesis.

Project Description

Objectives

The objectives of this project are to understand the basic etiology of autoimmune inflammation including uveitis and to find possible treatments for human uveitis.

Methods

The conventional methods for analysis of proteins and nucleic acids used include the following: protein purification; RNA and DNA isolation, characterization, and sequencing; molecular cloning; screening of clones; *in situ* hybridization; immunocytochemistry; and chromosome mapping. We also have synthesized and used oligopeptides and oligonucleotides. Bovine, murine, primate, and human materials are used. Animal experiments are carried out with Lewis rats and monkeys. T-cell response and adoptive transfer are done with lymph node or spleen cells of rat.

Major Findings

1. Local sequence homology was found between peptide M and several other foreign proteins, including potato proteinase inhibitor IIa, *Escherichia coli* hypothetical protein, hepatitis B virus probable DNA polymerase, Moloney murine sarcoma virus gag-polyprotein, Moloney murine leukemia virus gag-pol polyprotein, Baboon endogenous virus gag-pol polyprotein, and Baker's yeast histone H3.

2. The synthetic peptides of the above-mentioned proteins induced experimental autoimmune uveitis (EAU) in Lewis rats; its pathology was similar to that of EAU induced by peptide M or native S-antigen (S-Ag).

3. For the first time we proposed and showed the evidence that molecular mimicry plays a role in the process of pathogenesis of EAU and perhaps in autoimmune diseases in general.

4. Oral administration of histone H3 peptide suppressed EAU in the Lewis rats.

5. The suppression of EAU by histone H3 also was found in the EAU induced by the S-Ag. Thus, the tolerance also cross-reacted with the peptide, which has molecular mimicry.

6. The T-lymphocytes obtained from rats immunized with peptide M or yeast histone H3 transferred disease (i.e., EAU) in the naive rats (adoptive transfer) when stimulated either with peptide M or histone H3. In addition, oral tolerance was adoptively transferred from rats fed peptide M or histone H3 to the naive rats.

7. Infection by microorganisms which have cross-reactive antigens can break immune tolerance to a self-antigen and induce inflammatory autoimmune diseases.

Significance to Biomedical Research and the Program of the Institute

Uveitis is a leading cause of visual handicap in the United States and throughout the world. For many decades, physicians have suspected some types of uveitis to be induced by bacterial and viral infections; however, there is no clear link between infection and disease.

Autoimmune processes are thought to play a significant role in the pathogenesis of disease. Molecular mimicry—a process by which an immune response, directed against a nonself protein, cross-reacts with a normal host protein—may play a role in autoimmunity. Here we have proposed the idea of molecular mimicry and showed evidence that molecular mimicry may play a role in the pathogenesis of EAU. In addition, we have provided evidence that infection is a possible cause of autoimmune inflammation. These findings provide an important clue for understanding the etiology of autoimmune inflammatory diseases in human.

Proposed Course

The following studies are in progress or proposed for Fiscal Year 1993:

1. We will conduct further evaluation of foreign proteins similar to S-Ag that induce EAU.

2. We will characterize peptide M with respect to the minimum number of amino acids required for induction of EAU.

3. We will study the induction of EAU in transgenic mice that express foreign proteins in photoreceptor cells.

4. We will further characterize molecular mimicry and its role in EAU and human uveitis.

NEI Research Program

Retinal Diseases—Inflammatory Diseases

Publications

Chan CC, Li Q, Kikuchi T, Shinohara T, Nussenblatt RB: Enhancement of S-antigen and its mRNA in the irides of uveitic patients. *J Autoimmun* 5:719-732, 1992.

Eto K, Suzuki S, Singh VK, Shinohara T: Immunization with recombinant Escherichia coli expressing retinal S-antigen induced experimental autoimmune uveitis (EAU) in Lewis rats. *Cell Immunol* 147:203-214, 1993.

Hamasaki DI, Sato H, Santhanakrishnan S, Shinohara T: Correlation between the physiological and morphological changes in the experimental autoimmune uveitis induced by peptide G of S-antigen. *Exp Eye Res*, in press.

Nityanad S, Singh VK, Shinohara T, Paul AK, Singh VK, Agarwal PK, Agarwal SS: Cellular immune response of patients with uveitis to peptide M, a retinal S-antigen fragment. *J Clin Immunol*, in press.

Sunil S, Eto K, Singh VK, Shinohara T: Oligopeptides of three to five residues derived from uveitopathogenic sites of retinal S-antigen induce experimental autoimmune uveitis (EAU) in Lewis rats. *Cell Immunol* 148:198-207, 1993.

Laboratory of Sensorimotor Research

Report of the Chief, Laboratory of Sensorimotor Research

Robert H. Wurtz, Ph.D.

One of the most admired human abilities is that of skilled motor control—be it hitting a baseball in Baltimore or returning a tennis serve on Long Island. These abilities are highly sophisticated sensory motor tasks; they depend heavily on vision. The Laboratory of Sensorimotor Research concentrates on such sensory motor tasks, particularly in relation to the visual control of eye movements. Our goal is to understand the systems within the brain that process visual information and produce these eye movements and to understand what happens when disease or trauma leads these to fail. While our main interests are the systems in humans, we are fortunate to have a superb animal model, the Rhesus monkey, which allows us to explore not only the exact behavioral mechanisms related to visual motor behavior but also the underlying brain mechanisms controlling such behavior. Our investigations are best illustrated by a selection from the work of each of the five sections within the Laboratory.

Section on Oculomotor Control

One of the most frequent uses of vision for the control of eye movement is in the generation of rapid or saccadic eye movements—those eye movements that move the eyes from one part of the field to another. Such shifts allow us to look from one area of the field of interest to another. Both eyes move together to maintain binocular alignment, which is critical for good depth vision. Dr. Fred Miles and his collaborators are able to measure these eye movements with great accuracy in both humans and monkeys to determine how we solve a problem in generating these saccades (i.e., what happens when humans or monkeys are first confronted with images that differ in size for the two eyes). Such a difference in image size results when spectacle lenses cause the two eyes to see images that differ in size by several percent for each diopter of difference in correction between the eyes.

It previously has been shown that humans who wear spectacle lenses are able to generate saccades that differ in amplitude between the two eyes exactly as required by the different magnifications of the lenses, and usually it has been assumed that this ability results from some neural adaptive mechanism that adjusts for this over time. Dr. Miles' group has found that such an adaptation period is not necessary. These investigators found that humans immediately adjusted the amplitude of the eye movement in ways appropriate for the size of the stimulus. They hypothesize that it is not adaptation that is controlling binocular alignment of the eyes in this case but rather the use of the horizontal disparity in the image detected by the visual system. They were able to show exactly the same phenomena in the monkey, opening the way for extensive quantitative analysis of the parameters controlling these saccadic eye movements and the possibility of determining the brain mechanisms underlying this control.

Section on Visuomotor Integration

Another case illustrating the strong visual control of movement is from the work that my collaborators and I have done on the control of our movements through the environment and the stabilization of our posture. It has been shown in humans that motion through the visual field produces a specific pattern of large field visual motion, referred to as "optic flow." The nature of this large field visual motion is thought to provide information about our direction of movement through the environment. It also provides information to control our posture; humans sway back and forth substantially more with their eyes closed than with their eyes open.

In the past year we have tested whether monkeys use such visual information to control posture by training the monkey to stand on a small platform that measures how much the monkey sways and in what direction. By projecting onto a screen a pattern of

motion simulating the motion that would occur as the monkey leaned forward or back or side to side, we have been able to measure the monkey's postural changes. We have shown that the monkey responds to this visual stimulation and that the response is, in most respects, similar to that reported for humans. This now provides us with the ability to investigate further the regions of the brain that we know process this type of visual motion and to see whether alterations of these regions alter the monkey's use of the visual stimulation for the control of posture.

Section on Neural Modeling

The way neurons convey visual information has been studied by Dr. Lance Optican and his collaborators over the past several years. While in most studies of neurons within the brain the neuronal activity has been taken as the total number of action potentials or spike discharges emitted in response to a visual stimulus, Dr. Optican has shown that more information is conveyed if one looks at the temporal patterning of the action potentials as well as their total number. An understanding of this extra visual information may lead to a better understanding of visual perception. Dr. Optican and his collaborators previously have shown that neurons in a number of visual areas (i.e., V1, V2, V3, V4, and the inferior temporal cortex) both encode and transmit information about patterns that vary in form, color, brightness, and duration by a temporal code that represents these stimulus-dependent messages. In their present experiments, Dr. Optican's group trained monkeys to choose a particular stimulus according to whether it matched a previously given cue stimulus. They found that the temporal pattern of cell discharge varied with not only the stimulus falling on the receptive field of the cell but also with the nature of the cue stimulus. Furthermore, they found that each stimulus could be represented as the product of two wave forms that were specific for the features paired in each stimulus—for example, color and pattern. Thus, unique codes for every possible combination of visual features are not required. These experiments address the major issue in understanding information processing within the brain—the code by which this information is transmitted. This work clearly shows that neurons convey information about visual features by using a temporal code. This work may provide a key to understanding how the brain processes information to form a visual perception.

Section on Visual Behavior

Determining which visual stimulus is of importance for visual processing is one of the critical functions of the visual system. We look at only one part of the visual field at a time; the selection of the region of the visual field to look at next is the function referred to as "selective visual attention." Dr. David Lee Robinson has continued to explore the neurons in the brain that give evidence of participating in this attention process. He and his colleagues have conducted experiments on the superior colliculus to understand its role in visual attention. They have discovered that neurons there discharge at the appearance of certain visual stimuli and that these signals help indicate a change in the direction of attention. Other cells located in parts of the colliculus that are connected to the fovea also respond to visual stimuli, and here their activity starts the engagement of attention by images on the fovea. These are the first data to demonstrate a visual function of the superior colliculus that is not related to eye movements.

Section on Neuro-Ophthalmologic Mechanisms

One of our most effective methods of studying the systems within the brain is the alteration of that system by electrical stimulation or chemical injection (as in Dr. Robinson's experiments). Such modification allows us to test hypotheses about the contribution of a given set of cells within the system to the brain's function in controlling behavior. Dr. Michael Goldberg and his collaborators recently have been able to use such a technique, not only in the monkey model of the control of eye movements but also in humans. Dr. Goldberg previously had shown the characteristics of cells in a part of the frontal cortex of the monkey referred to as the frontal eye fields. Recent work using PET scanning identified the approximate region in the human where such frontal eye fields are located. By using the technique of focal magnetic stimulation, which changes the

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00256-05 LSR

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Information Processing by Visual System Neurons

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, end institute affiliation)*

PI:	Lance M. Optican	Ph.D.	Chief, Neural Modeling Section	LSR, NEI
Others:	John W. McClurkin	Ph.D.	Staff Fellow	LSR, NEI
	Arthur V. Hays	B.A.	Electronics Engineer	LSR, NEI
	Brad J. Zoltick	M.A.	Computer Programmer	LSR, NEI
	Jennifer A. Zarbock	B.A.	Electronics Engineer	LSR, NEI
	Merk Na Chee-Orts	Ph.D.	Visiting Associate	LSR, NEI
	Marc H. Cohen	M.S.E.	Visiting Associate	LSR, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Sensorimotor Research

SECTION

Neural Modeling Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.6	4.0	1.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Our studies indicate that different visual areas in the brain may communicate via temporally modulated messages. We showed previously that neurons in different areas of the brain encode and transmit information about stationary, two-dimensional pictures that vary in form, brightness, and duration. We also showed that information about remembered visual features was carried by a temporal code. Now we have extended those studies to show that neurons in the visual cortex (areas V1, V2, V3, and V4) carry information about the form, color, luminance, and size of a stimulus in a temporally modulated code. Our results suggest that cortical neurons are able to convey information about many different features without confounding them. The mechanism for encoding these multiple messages uses temporal modulation to multiplex the different messages on the neuron's response in a separable way.

electrical activity of the brain in a small region below the skull, they have been able to show that stimulation of the frontal eye field of humans alters saccadic eye movements. Furthermore, they have been able to show that the type of alteration depends on the time at which the stimulation is given before the onset of the saccade. Thus, identification of a region in the brain of the monkey has led to the localization, and now the modification, of a similar area in human subjects.

A part of the activity of the Laboratory this year involved the move of those laboratories devoted primarily to research on monkeys into the new Silvio O. Conte Building (Building 49). This move was completed by June 22. The new building provides superb facilities for nonhuman primates, fine laboratories for investigation, and a few small rooms for offices.

Project Description

Objectives

Perception and recognition of complex visual pictures depend on the normal function of interconnected brain regions extending from the retina through the inferior temporal cortex. The properties of these regions are derived from the function of the single neurons within them. Thus, to understand how visual perception occurs, we must learn how information is encoded by the neurons in each stage of processing. If we could understand this neuronal code, it might be possible to distinguish between information related to the physical properties of a stimulus (e.g., form, luminance, color, and size) and information related to its behavioral significance (e.g., leading to a reward).

Individual neurons in all the visual areas studied thus far (retinal ganglion cell fibers; lateral geniculate nucleus neurons; pulvinar neurons; cortical neurons in visual areas V1, V2, V3, and V4; and inferior temporal cortical neurons) encode and transmit information about stationary, two-dimensional pictures that vary in form, color, brightness, and duration. The neurons use a multidimensional temporal code to represent and transmit their stimulus-dependent messages. We now have shown that visual neurons convey complex messages about (1) a stimulus' physical parameters and (2) its behavioral significance. Using information theory, we can begin to explore how physical and behavioral components of a neuron's response contribute to higher visual cognitive functions such as perception, attention, and memory.

Major Findings

We have developed a new approach to studying single neurons in which they are treated as communication channels that transmit information about visual pictures in their responses. This has allowed us to apply methods from signal processing, statistics, systems analysis, and information theory to understand single neurons.

According to a commonly held view of neuronal function, the strength of a neuron's response represents how closely the stimulus matches the receptive field's characteristics (e.g., orientation or color). Thus, if response strength were the only parameter a neuron could use to encode information, different stimulus features would be confounded by individual neurons. Using informational analysis, we have shown that information about different stimulus parameters is not confounded but is carried across the different parts of the multidimensional neuronal code.

In recent experiments, we recorded responses of neurons in four visual cortical areas—V1, V2, V3, and V4—of a monkey trained to choose one of three parafoveal stimuli on the basis of whether their color or pattern matched that of a cue stimulus. These responses were modulated by the pattern and color of the stimulus on the receptive field and by the pattern or color of the preceding cue. In other experiments, stimuli consisted of either colored bars that were isoluminant with the background or black or white bars that varied in size. Information about stimulus features developed continuously, but not uniformly, throughout the time-course of the neuronal responses. Most of the information was encoded in the initial 50-60 msec of the response. Some neurons also encoded a large amount of information in a second 50-msec interval, beginning 20-30 msec after the first.

These results show that neurons in V1-V4 carry information about the color, pattern, contrast, and size of stimuli. Finally, the development of information over time in different areas suggests that temporally modulated waves of activity may form a code for visual information. In fact, the response to each stimulus could be represented as the product of two waveforms that were specific for the features paired in each stimulus (e.g., color and pattern, color and orientation, contrast and orientation, or size and orientation). Feature-specific waveforms for each color, pattern, contrast, orientation, and size were isolated from the neuronal responses by a neural net. The product of these feature waveforms predicted the neuronal responses to stimuli with feature combinations not used to train the neural net (e.g., novel-colored patterns).

Feature waveforms were often similar for all neurons within a cortical area. To compare these waveforms across cortical areas, we pooled all the responses from neurons within each area. Waveforms encoding pattern were strikingly similar across all areas, irrespective of the behavioral task. Waveforms encoding color in the color/pattern task differed between cortical areas, but there was a striking similarity for waveforms encoding color in the

isoluminant-color/orientation paradigm. These results suggest that neurons convey information about compound visual features by multiplexing feature-specific messages together. The invariance of the code waveforms suggests that information about a stimulus feature is represented similarly in all visual areas.

Significance to Biomedical Research and the Program of the Institute

This project studies how visual information is encoded and transmitted by neurons. Knowledge of these fundamental processes is important for understanding deficits of visual processing, such as those occurring in amblyopia, and for developing visual prosthetic devices to compensate for field defects or blindness.

Proposed Course

Discovering that the responses of visual system neurons are multidimensional led to the discovery that information about multiple stimulus features may not be confounded by single neurons, a result with important, even revolutionary, consequences. We now know that a substantial part of the temporal modulation arises after visual information has left the retina. Our latest results show that the neural code arises due to the influence of feedback.

Ever since we found evidence of a neural code and saw a possible structure for it, we have been trying to delineate it. The properties of the code should give clues about the functions performed by the neurons. Now that we have shown that some of the temporal codes are invariant across cells, and even across areas, a new theory of visual information processing is required. This theory will treat the visual system more as a concurrent processing system than as a hierarchical cascade of independent areas. Both these issues are being pursued.

In addition, our findings have suggested previously unconsidered principles as the basis for interactions among neurons. To investigate these principles, we need to collect and analyze data from several simultaneously recorded neurons. Thus, we have been developing the apparatus needed to make multiple, simultaneous single-neuronal recordings. The apparatus should be completed sometime during the next year. The simultaneously recorded responses will be related to each other through use of recent extensions to methods of signal identification, which should allow us to develop models that will describe, relatively rapidly, the roles of single neurons as components of larger networks. These studies should yield a better understanding of the information transmission mechanisms, such as pattern perception and recognition, used for cognitive functions.

Our findings suggest a completely new conceptional framework in which to investigate neuronal function. One presumed reason for the huge number of single neurons has been the necessity to unconfound stimulus features. However, we propose that the simultaneous messages about different features can be used as tags, so that the messages which arise in different processing regions of the visual system can be reunited into a unified percept. This would provide the mechanism to build a whole perception across many processing regions. With the use of new computational equipment, we are exploring this hypothesis both experimentally and theoretically.

NEI Research Program

Strabismus, Amblyopia, and Visual Processing—Visual Processing and Functional Organization (Structure and Function of Central Visual Pathways)

Publications

Chee-Orts MN, Optican LM: Cluster method for analysis of transmitted information in multivariate neuronal data. *Biol Cybern* 69:29-35, 1993.

Eskandar EN, Optican LM, Richmond BJ: Role of inferior temporal neurons in visual memory: II. Comparing temporal waveforms arising from vision and memory. *J Neurophysiol* 68: 1296-1306, 1992.

Eskandar EN, Richmond BJ, Optican LM: Role of inferior temporal neurons in visual memory: I. Temporal encoding of information about visual images, recalled images, and behavioral context. *J Neurophysiol* 68:1277-1295, 1992.

Kapoula Z, Robinson DA, Optican LM: Visually induced cross-axis postsaccadic eye drift. *J Neurophysiol* 69:1031-1043, 1993.

McClurkin JW, Zarbock JA, Optican LM: Temporal codes in monkey striate cortex for colors, patterns and memories, in Peters AA, Rockland KS (eds): *Primary Visual Cortex of Primates, Cerebral Cortex.* New York, Plenum, 1993, vol 10, in press.

Zee DS, FitzGibbon EJ, Optican LM: Saccade-vergence interactions in humans. *J Neurophysiol* 68:1624-1641, 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00049-15 LSR

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cerebral Cortical Mechanisms for Eye Movements and Visual Attention

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Michael E. Goldberg	M.D.	Chief, Neuro-Ophthalmologic Mechanisms Section	LSR, NEI
Others:	Edmond J. FitzGibbon	M.D.	Medical Officer	LSR, NEI
	Carol L. Colby	Ph.D.	Senior Staff Fellow	LSR, NEI
	Suzanne Y. Musil	Ph.D.	Staff Fellow	LSR, NEI

COOPERATING UNITS *(if any)*

Medical Neurology Branch, National Institute of Neurological Disorders and Stroke

LAB/BRANCH

Laboratory of Sensorimotor Research

SECTION

Neuro-Ophthalmologic Mechanisms Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.3	4.0	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Two lines of inquiry were followed to determine how the cerebral cortex and its efferent regions control eye movements and visuospatial attention.

In one, focal transcranial magnetic stimulation of the human frontal eye field was used to determine the effect that frontal eye field activity can have on the generation of saccadic eye movements. Depending on the relationship of the exogenous stimulation to the ongoing processes of saccade initiation, such stimulation can facilitate or interfere with saccade generation.

In the other, single neuron recording was used to probe the mechanisms whereby the parietal cortex of the monkey achieves spatial accuracy. Neuronal behavior in a double-step task is entirely predicted by presaccadic and predictive-shift activity in a single-step task. The activity of parietal neurons is most consistent with their specifying a shift of visual attention of particular amplitude and direction.

Project Description

Objectives

This section has concentrated on two aspects of the physiology and phenomenology of higher visual and oculomotor processing in the monkey and man, especially as these functions relate to the parietal and frontal regions of the cerebral cortex, their afferent regions, and their efferent targets. Previous work in this laboratory has shown that neurons in the parietal cortex have neurons that discharge in response to visual stimuli and before saccadic eye movements. There is a predictive aspect of these neurons' visual responses: Neurons respond to stimuli in which spatial location will be brought into their visual receptive fields by impending saccadic eye movements.

The double-step task of Hallett and Lightstone has been considered the paradigmatic example of accurate spatial behavior. Humans and monkeys perform this task accurately, making accurate eye movements to successive, briefly flashed targets, despite a dissonance between the retinal location of the target and the amplitude and direction of the saccade necessary to fixate it. Work in the laboratory during this year has concentrated on comparing the activity of neurons in the double-step task to see whether that activity could be explained by their activity in more simple tasks.

We have demonstrated a neuronal mechanism for the initiation and targeting of saccadic eye movements in the monkey frontal eye field, where neurons discharge predictively before purposeful saccades and intracortical electrical microstimulation elicit saccades. In this period, we used the technique of transcranial magnetic stimulation in human subjects to stimulate the frontal eye field selectively at various times in the generation of visually guided saccades in order to see whether electrical stimulation of this region affected saccades in a manner consonant with the single-neuron data from the monkey.

Methods

Monkeys were implanted with magnetic search coils for the measurement of eye position, along with devices for temporary restraint and electrophysiological recording and stimulation. The monkeys were trained to perform a number of visuomotor tasks, including fixation, saccades, and smooth pursuit. Microelectrodes placed in the lateral intraparietal area single neurons enabled study while the monkey performed various visuomotor tasks.

Normal volunteers were instructed to fixate a central target and make a saccade as quickly as possible in response to the appearance of a peripheral light. Eye movements were measured by an infrared eye tracker. In randomly interleaved trials, focal transcranial magnetic stimulation was delivered through a figure-eight-shaped coil over the presumed site of the frontal eye field, which was located with reference to the hand motor representation.

Major Findings

Transcranial magnetic stimulation applied long before the onset of the expected saccade produced shorter saccadic reaction times and increased saccade acceleration. Conversely, transcranial magnetic stimulation applied shortly before the onset of the expected saccade yielded longer saccadic reaction times and decreased saccade acceleration. Similar effects were observed when subjects were instructed to perform antisaccades. Independent of the effect on saccadic reaction times, transcranial magnetic stimulation produced transient divergence of the eyes immediately preceding saccade onset. When transcranial magnetic stimulation occurred during an ongoing saccade, it transiently arrested or slowed the eye movement. In summary, transcranial magnetic stimulation can facilitate or retard saccadic reaction time and can affect the metrics of saccadic eye movements. When it occurs at a time at which it might interfere with ongoing frontal eye field processing, it slows saccades. When it appears at a time at which it could reasonably be expected to substitute for normal processing, it speeds up saccadic reaction times.

Neurons in the lateral intraparietal have visual and sometimes presaccadic responses. The visual responses are sometimes predictive, occurring before a saccade that will bring the spatial location of a visual target into the neuron's receptive field. Neurons that discharge in the double step tasks have presaccadic responses, predictive responses, or both. The activity in the double-step task approximates the sum of the presaccadic and predictive response. These data illustrate that the response in the double-step task emerges as a consequence of the neuron's response in relation to single eye movements and that it is

unnecessary to postulate a special mechanism to account for activity in the double-step task.

Significance to Biomedical Research and the Program of the Institute

Understanding the way in which the cerebral cortex and its afferent regions guide eye movements and modulate visual attention and learning is useful as a model for the neural control of other, more complicated behaviors. It is also a key to understanding and developing treatments for disorders of the neural control of vision, eye movements, and attention.

Proposed Course

The frontal eye fields will be examined to see whether they have predictive responses. The activity of neurons in both the frontal eye fields and the parietal cortex will be examined under the more natural condition of visual search.

NEI Research Program

Strabismus, Amblyopia, and Visual Processing—Visual Processing and Functional Organization (Structure and Function of Central Visual Pathways)

Publications

Colby CL, Duhamel JR, Goldberg ME: The analysis of visual space by the lateral intraparietal area of the monkey: The role of extraretinal signals. *Prog Brain Res* 95:307-316, 1993.

Colby CL, Duhamel JR, Goldberg ME: Ventral intraparietal area of the macaque: Anatomic location and visual response properties. *J Neurophysiol* 69:902-914, 1993.

Duhamel JR, Goldberg ME, FitzGibbon EJ, Sirigu A, Grafman J: Saccadic dysmetria in a patient with a right frontoparietal lesion: The importance of corollary discharge for accurate spatial behavior. *Brain* 115:1387-1402, 1992.

Goldberg ME, Musil SY, FitzGibbon EJ, Smith MK, Olson CR: The role of the cerebellum in the control of saccadic eye movements, in Mano N (ed): *Cerebellum and Basal Ganglia in the Control of Movement.* Amsterdam, Elsevier, 1993, in press.

Olson CR, Musil SY, Goldberg, ME: Superior cingulate cortex and visuospatial cognition: Properties of single neurons in the behaving monkey, in Vogt BA, Gabriel M (eds): *The Neurobiology of Cingulate Cortex and Limbic Thalamus.* Boston, Birkhauser, 1993, in press.

Segraves MA, Park K: The relationship of monkey frontal eye field activity to saccade dynamics. *J Neurophysiol* 69:1880-1889, 1993.

Stanton GB, Bruce CJ, Goldberg ME: Topography of projections to the frontal lobe from macaque frontal eye fields. *J Comp Neurol* 330:286-301, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00153-11 LSR

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visual Motion and the Stabilization of Gaze

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Frederick A. Miles	D.Phil.	Senior Research Physiologist	LSR, NEI
Others:	Urs Schwarz	M.D.	Visiting Associate	LSR, NEI
	Claudio Busettini	Ph.D.	Visiting Fellow	LSR, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Sensorimotor Research

SECTION

Oculomotor Control Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	1.3	1.2

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It has been known for some time that corrected anisometropes who wear spectacle lenses of different power in front of the two eyes are able to generate saccades that differ in amplitude in the two eyes exactly as required by the differing magnifications of the spectacle lenses. A common assumption is that this ability results from the operation of a neural adaptive mechanism, which, over time, gradually adjusts the relative amplitudes of the saccades produced by the two eyes. We now report that when the scenes viewed by the two eyes suddenly differ in size, the two eyes produce saccades that immediately differ in amplitude without any prior period of adaptation. Two slide projectors and an arrangement of orthogonal polarizing filters were used to present overlapping stationary random dot patterns simultaneously yet separately to the two eyes. The right eye always saw the same pattern while the left eye saw a pattern that was initially identical (pretest) and later replaced by one that was 8% smaller (test). The positions of both eyes were recorded with the electromagnetic search coil, and horizontal saccades were elicited by target spots projected onto the pattern through polarizing filters so as to be visible only to the right eye. Subjects were three humans and three rhesus monkeys. Immediately upon viewing the smaller pattern, the left eye produced horizontal saccades that were significantly smaller than those produced by the right eye. This indicates that the saccadic system has some ability to cope immediately with aniseikonia, the compensation being almost complete in some subjects. We suggest that the important cue in these experiments is horizontal disparity and that the saccadic system uses this to scale the relative amplitudes of the saccades produced by the two eyes.

Project Description

Objectives

In recent years there has been considerable interest in the binocular alignment of the two eyes because it is critical for good stereoscopic depth vision. The advent of new techniques for recording eye position with high precision in both monkeys and humans has, for the first time, permitted detailed studies of the relative alignment of the two eyes on objects at varying distances in a three-dimensional world. We have used the high-resolution electromagnetic search coil to examine the saccadic eye movements of both monkeys and humans when they are first confronted with images that differ in size for the two eyes.

Correction of anisometropia with spectacle lenses causes the two eyes to see images that differ in size by 2-3% for each diopter of difference. It has been previously shown by others that human subjects who wear such spectacles are able to generate saccades that differ in amplitude between the two eyes exactly as required by the differing magnifications of the spectacle lenses. It has been commonly assumed that this ability results from the operation of a neural adaptive mechanism that over time gradually adjusts the relative amplitudes of the saccades produced by the two eyes. However, previous investigators had not recorded eye movements immediately after the subjects put on the spectacles; hence, they did not know whether this behavior was immediate or required a period of adaptation. Therefore, we looked at the saccadic eye movements produced by both human and monkey subjects when first confronted with the challenge of aniseikonia (i.e., unequally sized images seen by the two eyes).

Methods

The subjects (i.e., three humans and three rhesus monkeys) faced a tangent screen onto which were projected two superimposed images, each of which was visible to only one of the two eyes. This we achieved using a special screen and polarizing filters so that one of the images was polarized in a plane orthogonal to the other. When viewing the screen through goggles with cross-polarizing filters, each eye could see only one of the images, which were computer-generated random dot patterns. We recorded the positions of both eyes using the electromagnetic search coil method while the subjects made saccadic eye movements between target spots projected onto the screen through polarizing filters so as to be visible only to the right eye. The targets were located 10 degrees to the right and left of straight ahead. The right eye always saw the same random dot pattern, whereas the image seen by the left eye could be either the same or 8% smaller, resulting in a gradient of binocular disparities across the scene (i.e., slight differences in the locations of the images on the two retinas). Fifty leftward and fifty rightward saccades were recorded when the patterns seen by the two eyes were identical (pretest) and another 50 each when the two patterns differed in size (test).

Major Findings

When the patterns were identical in size, the two eyes made saccades that were essentially identical in amplitude, though not velocity. Immediately on viewing the smaller pattern, the left eye produced horizontal saccades significantly smaller than those produced by the right eye. Similar observations were made using scenes that differed only in their horizontal dimensions, when the pattern of disparity was like that experienced by an observer who views a vertical surface slanting away from him or her. A striking feature of such stimuli was that, at best, the human observers had only a very weak perception of such a slanting surface. However, the horizontal saccades of all subjects immediately showed considerable compensation for the aniseikonia; in some subjects it was almost complete. For example, when the left eye saw a pattern that was 8% smaller horizontally, the saccadic amplitude ratios (left eye/right eye) of the three humans during the test period were on average smaller than those during the pretest by 7.3%, 4.0%, and 7.3% for rightward saccades and by 5.5%, 3.8%, and 7.4% for leftward saccades. Similar saccadic data were obtained from the three rhesus monkeys.

Significance to Biomedical Research and the Program of the Institute

These data indicate that, when suddenly confronted with aniseikonic images, the saccadic systems of both monkeys and humans are able to make saccades that differ appropriately in size. Furthermore, in the experiments, the only cue provided was (horizontal) disparity, indicating that the saccadic system can directly utilize such cues to adjust the relative amplitudes of the saccades produced by the two eyes.

It also is interesting that the motor system responded to the disparity cues, even though human subjects failed to perceive them.

Proposed Course

Future experiments will further examine the saccadic eye movements associated with aniseikonia in both human subjects and monkeys. The research will involve parametric studies of these asymmetric saccades to establish the limits of the system and their impact on saccadic dynamics.

NEI Research Program

Strabismus, Amblyopia, and Visual Processing—Image Formation and Stabilization (Ocular Motility)

Publications

Kimmig HG, Miles FA, Schwarz U: Effects of stationary textured backgrounds on the initiation of pursuit eye movements in monkeys. *J Neurophysiol* 68:2147-2164, 1992.

Miles FA: The sensing of rotational and translational optic flow by the primate optokinetic system. *Rev Oculomot Res* 5:393-403, 1993.

Miles FA, Busettini C, Schwarz U: Ocular responses to linear motion, in Shimazu H, Shinoda Y (eds): *Vestibular and Brain Stem Control of Eye, Head and Body Movements.* Tokyo, Japanese Scientific Societies Press/Karger 1992, pp 379-395.

Miles FA, Wallman J: Prologue. *Rev Oculomot Res* 5:v-viii, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00045-15 LSR

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visuomotor Properties of Neurons in the Thalamus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	David Lee Robinson	Ph.D.	Section Chief	LSR, NEI
Others:	Alexander A. Kustov	Ph.D.	Fogarty Fellow	NEI

COOPERATING UNITS *(if any)*

Department of Anatomy, Howard University (Robert J. Cowie, Ph.D.)

LAB/BRANCH

Laboratory of Sensorimotor Research

SECTION

Visual Behavior Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.4	1.0	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Visual stimuli excite the retina and often produce a shift of attention. To understand some of the brain mechanisms involved in shifts of attention, we recorded the activity of neurons in the superior colliculus while monkeys performed various tasks. We discovered that neurons in the representations of the peripheral visual fields respond uniformly to targets, regardless of the direction of the animal's attention. This occurs whether attention is shifted by visual cues or by more cognitive processes. In contrast, neurons within parietal cortex are modulated by the direction of the animal's attention. Neurons within the foveal representation of the colliculus respond differentially to fixation targets, depending on the behavioral task. While the animal is simply fixating, these neurons are only weakly responsive; during more attention-demanding tasks, there are more brisk responses of these same cells. We injected the colliculus with muscimol, a GABA-agonist, and discovered that it produced a slowing of responses to all targets within the injected visual field. The neurons recorded at the injection site had increased spontaneous activity and were also more responsive to visual stimuli. These data are some of the first to demonstrate an attentional contribution of the colliculus, independent of eye movements. The results suggest that the superior colliculus provides a visual trigger signal for shifts of attention.

Project Description

Objectives

Visual stimuli continually excite the eye, and some of these elicit a shift of attention. The present studies were conducted to understand some of the mechanisms the brain uses to mediate attentional shifts. We sought to discover the physiological mechanisms used within the superior colliculus to produce visual activity that initiates shifts of attention. In addition, we sought to learn the functional contributions this collicular activity might make to the whole organism. Because we previously had studied the contributions of pareital cortex to attention, this study was intended to compare these two areas.

Methods

To study the activity of the superior colliculus during attentional behavior, we trained monkeys to enter a primate chair, sit quietly, fixate spots of light, and make eye movements to them. After preliminary training, the monkeys were implanted with several recording devices during sterile surgery. Scleral search coils were implanted in the monkeys for recording eye movements and eye position. The monkeys learned to contact a bar at the beginning of each trial. They then fixated on a spot of light projected onto a screen and released the bar whenever target lights were flashed onto the screen. The monkeys also learned to make eye movements to spots of light flashed onto the screen, as well as make fine discriminations of selected fixation targets. At the sites of interesting cellular activity, small marking lesions were made for later localization on histological sections.

Major Findings

As we previously had demonstrated, monkeys respond faster to visual targets that are preceded by visual cues at the same location than to visual targets that follow cues at other points. It is hypothesized that the cue shifts attention to that point and thereby improves visual performance. We studied neurons within the superficial layers of the superior colliculus while monkeys performed this and related tasks.

Collicular neurons compose a uniform population of cells with consistent latencies and response magnitudes. Data from our previous studies of parietal cortex show that cells in that region make up at least three subgroups; most of these neurons could be driven by our collicular afferents. All collicular neurons responded to both the onset and offset of visual cues that controlled the monkeys' attention. In addition, the responses to these cues were uniform across the temporal intervals used. When these cues excited collicular neurons, they produced a very stong period of refractoriness that was much more intense than the one measured for parietal cells.

When collicular neurons were tested with the attentional cue outside the visual receptive field but positioned to produce attentional effects, collicular cells responded equivalently to all targets, regardless of where attention was directed. Cells in parietal cortex tested under these same conditions were differentially modulated, depending on the direction of the animals' attention. When collicular neurons were tested via tasks that cognitively controlled the direction of the animals' attention, there also was consistent response independent of the attentional direction.

We also studied neurons within the foveal representation of the colliculus. When these cells were analyzed at the time that attention was shifted to the cue, there were no changes in the activity levels of the cells. These data suggest that the foveal parts of the colliculus do not participate in the shifting of attention. However, the cells had different activity patterns, depending on the behavioral task. When the animals simply had to fixate a spot of light to obtain a reward, there was only weak responding to the onset of the fixation point. These cells were excitable by other lights, so there was no overall suppression of their excitability. When the animal fixated the identical light during the performance of the attentional cuing task, there was a much stronger response from collicular neurons. The cells responded as if the act of attending had facilitated their visual responsiveness. Comparably strong responses were obtained when the animal actively attended to the same fixation point during a special foveal attention task. These data suggest that the foveal region of the colliculus is under attentional control, enhancing visual excitability, whereas the peripheral collicular representation is not attentionally modulated.

To evaluate the contribution of these collicular signals to the monkeys' behavior, we altered collicular activity by microinjections of muscimol, a

GABA-agonist. After an injection of muscimol, the monkeys were slow to respond to all visual targets that appeared within the affected region of visual space. However, collicular neurons at the site of the injection were more responsive after the injections, contrary to observations obtained from microiontophoresis. Now there was an increase in spontaneous activity of the collicular neurons. In addition, these cells discharged more intensely to the cues and targets. No changes in cellular activity were observed with injections of saline.

These data suggest that the effects of limited injections of muscimol are not always inhibitory and do not decrease transmission through a structure. They also suggest that the discharge of collicular neurons provides a visual trigger signal that can lead to a shift of attention. When this signal is modified, in this case by the production of increasing and distracting discharges, there is a generalized breakdown in performance.

Significance to Biomedical Research and the Program of the Institute

Various disorders of the brain produce abnormal attention and eye movements. Some of these, such as progressive supranuclear palsy, involve dysfunction of the superior colliculus. The data obtained this year help to define the contribution of the colliculus to these symptoms. Understanding these processes might facilitate early detection of such disease processes. For rehabilitation of people with damage to parts of the brain, it is important to know the normal capacities of certain neural centers and the ways in which one brain area can take over the functions of damaged areas. By gaining a clearer understanding of the contribution of the colliculus to visual behavior, we can gain insights into its capacity to acquire other functions.

Proposed Course

One of the major interests in this Section is visual attention. We recently have discovered that saccadic eye movements can be initiated at very short latencies. Under certain conditions, including manipulation of the state and direction of attention, saccadic eye movements can begin rapidly. These movements are termed "express saccades." Whereas our previous studies have demonstrated that certain regions of the parietal cortex and pulvinar are related to visuospatial attention, our future studies will attempt to determine the contributions of the pulvinar and parietal cortex to express saccades. Rhesus monkeys will be trained on a variety of eye movement tasks that will reliably evoke express saccades. Subsequently, we will electrically excite or chemically inactivate these portions of the brain to determine how their attentional mechanisms contribute to the initiation of saccadic eye movements.

NEI Research Program

Strabismus, Amblyopia, and Visual Processing—Visual Processing and Functional Organization (Structure and Function of Central Visual Pathways)

Publications

Bowman EM, Brown VJ, Kertzman C, Schwarz U, Robinson DL: Covert orienting of attention in macaques. I. Effects of behavioral context. *J Neurophysiol* 70:431-443, 1993.

Brown VJ, Schwarz U, Bowman EM, Fuhr P, Robinson DL, Hallett M: Dopamine dependent reaction time deficits in patients with Parkinson's disease are task specific. *Neuropsychologia* 31:459-469, 1993.

Robinson DL: Functional contributions of the primate pulvinar. *Prog Brain Res* 95:371-380, 1993.

Robinson DL, Cowie RJ: Attentional engagement and the pulvinar. *Behav Brain Sci*, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00109-13 LSR

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visuomotor Processing in the Primate Brain

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert H. Wurtz	Ph.D.	Chief	LSR, NEI
Others:	Charles J. Duffy	M.D., Ph.D.	Staff Fellow	LSR, NEI
	Hiroshi Aizawa	Ph.D.	Visiting Fellow	LSR, NEI
	Gregg H. Recanzone	Ph.D.	Guest Researcher	LSR, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Sensorimotor Research

SECTION

Visuomotor Integration Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	1.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Saccadic or rapid eye movements shift the direction of gaze from one part of the visual field to another, but most of normal vision occurs during the period of visual fixation between these saccades. This year we tested the hypothesis that fixation cells in the rostral superior colliculus (SC) suppress the activity of saccade-related cells in the posterior colliculus and thus also suppress saccades. Electrical stimulation of fixation cells interrupted saccades and the burst of activity in the posterior colliculus that precedes saccades. Stimulation of the rostral pole also lengthened the interval between a series of saccades that result from stimulation of the posterior SC. Both results are consistent with the hypothesis that the fixation cells in the rostral SC regulate the generation of saccades.

As we move through the environment, we generate a full-field visual motion—a pattern of optic flow. We have previously studied cells in the cerebral cortex of monkey that appear to be selective for such flow. This year we began experiments to test the extent to which monkeys respond to this visual flow stimuli. When the monkey stood on a platform that allowed postural changes, we could detect in the monkey a sway related to the optic flow. Vibration of the platform increased reliance on flow. These experiments demonstrate the sensitivity of the monkey to flow stimuli and open the way to testing the effect of removal of the flow sensitive cortical neurons on the use of optic flow stimuli.

Project Description

Objectives

Saccadic eye movement shifts the eyes rapidly from one item of interest in the visual field to another. The neuronal organization underlying this system has been studied intensively in the monkey during the past 20 years, particularly in this Laboratory. Our work this year centered on a brainstem structure that is related to the generation of these saccadic eye movements—the superior colliculus. As we move through the environment, we use the resulting motion of the visual field as a whole, referred to as "optic flow," to provide stabilization of posture and possible guidance of movement. We had previously studied the visual processing related to such control in an area of the cerebral cortex, the medial superior temporal region of the superior temporal sulcus. Our experiments this year concentrated on the demonstration of the use of these stimuli by the monkey.

Methods

The Rhesus monkey, *Macaca mulatta*, is an incomparable model of the visual system of humans. Its saccadic eye movements are nearly identical to those of humans. The response of cells to optic flow field stimulation suggests that the monkey also uses such full-field stimulation in its behavior, although this is less well understood. In the study of saccadic eye movements, the monkey is awake and trained to perform a visual motor task that involves making saccadic eye movements from one target to another. Because the cooperation of the monkey is required to obtain a high level of visual performance, the monkey is rewarded for making these eye movements.

The entire experiment is managed by an online computer that turns on the stimuli, rewards the monkey, collects the neuronal and behavioral data, and stores that data on magnetic disks for later computer analysis. In the experiment on the effects of full-field visual stimulation, the monkey is free to move within a cage; his posture is measured by strain gauges attached to a flat platform on which the monkey is trained to stand. Full-field visual stimulation is projected on one side of the cage, which the monkey faces.

Major Findings

We had determined previously that damage to the superior colliculus produces immediate deficits in the generation of saccadic eye movements. We infer, therefore, that cells within the colliculus are related to the generation of saccades. This year we completed a detailed study of the types of cells within the superior colliculus. We had detailed the two types of cells related to the generation of saccades—(1) burst cells and (2) preparatory or buildup cells—in last year's annual report. This year we completed experiments and analysis on the remaining major cell type within the superior colliculus—fixation cells. These cells do not discharge with a saccadic eye movement. Rather, they increase their discharge rate when the monkey actively fixates on an object and decrease their discharge during saccadic eye movements. The period of this decrease is roughly equivalent to the duration of the saccadic eye movement, suggesting that the pause of these cells is necessary for the generation of the saccadic eye movement.

Two studies demonstrated that this interaction between fixation cells and saccade-related cells does occur. In the first, we electrically stimulated the fixation cells while recording both the eye movement and discharge of the saccade-related cells within the colliculus. We found that the stimulation of the fixation zone interrupted the saccadic eye movement and reduced the discharge of the saccade-related cells. The reduction in activity, a presumed indication of inhibition, was greater for the burst cells than for the buildup cells. This action on the saccade cells is consistent with our hypothesis that the fixation cells within the colliculus act to inhibit the generation of saccadic eye movements while the monkey is actively fixating.

The second set of experiments on the action of the fixation cells was to test the effect of their stimulation on the generation of a "staircase" of saccades. This staircase occurs when the saccade-related cells of the superior colliculus are stimulated over a period of several hundreds of milliseconds. The result of this stimulation is a series of individual saccades with a pause between each saccade.

Why there would be such a series of identical saccades rather than one saccade alone has been a puzzle for more than 20 years. One explanation might be that, when the saccade-related cells are stimulated, they are active in the absence of a pause in the fixation cells such that the eye is not held in its new position after the saccade by the activity of the fixation cells. To test this notion, we electrically stimulated the fixation zone to see whether it altered the time between saccades; it did. As we stimulated the fixation cells, we increased the period between successive saccades in the staircase, indicating that it was the activity in the fixation zone that determined the spacing between the saccades. This finding is also consistent with the hypothesis that the fixation cells, when active, inhibit the generation of saccadic eye movements.

Our second set of investigations on the effect of large-field visual stimulation has concentrated not so much on the activity of cells within this area but on the consequence of the visual stimulation to the monkey's behavior. One of the goals of our research is not only to determine the relationship of cell activity to visual stimulation and behavior but to determine whether removal of these cells leads to a change in visual motor behavior. Thus, in the current set of experiments, we have begun to test the effect of the large-field stimulation on the monkey's behavior in order to eventually test whether removal of these cells affects that behavior.

Our strategy has been to use the well-known effect of full-field visual stimulation on human posture. Whether such stimulation is used by monkeys has never been determined. We measured the monkey's posture while full-field stimulation was given by using strain gauges attached to a platform on which the monkey was trained to stand. We oscillated the full-field motion over a period of several seconds and observed a swaying motion of the monkey synchronous with the moving visual field. We were struck, however, that the monkey was able to compensate, using other mechanisms, so that repeated presentations of the visual stimulation no longer produced the sway.

One mechanism that could contribute to this compensation is proprioception, the sense that indicates the position of muscles and joints. To reduce this sensation in the monkey, we attached a low-frequency vibrator to the platform, thereby reducing the amount of information proprioception and increasing the monkey's sensitivity to visual stimulation. This increased sensitivity persisted over time. These experiments seem to show that (1) monkeys are sensitive to full-field stimulation, as are humans, and (2) the use of the visual stimuli is coupled with the use of proprioception, and probably vestibular sensation, to control posture.

Significance to Biomedical Research and the Program of the Institute

The saccadic eye movement system usually has been taken in isolation as a system that moves the eye from one point of the visual field to another. Our studies on the fixation cells within the superior colliculus and their effect on the generation of saccadic eye movements demonstrate that a system for visual fixation is as important as the generation of saccades. Thus, the saccade moves the eye from one point to another, and the fixation system holds the eye in position. All of our vision results from the period of fixation, so that understanding this fixation system is essential to understanding normal vision. Just as the study of this visual fixation is not generally studied in primates, it is not frequently studied in the clinic. We hope that clarification of the interaction of the saccadic and fixation systems in the monkey will stimulate analysis of fixation in the human as well.

The effect of full-field or optic flow stimulation studied in the monkey on postural responses attempts to establish the monkey as a model of the effect of vision on postural control. These experiments move the study of the monkey from one of just eye movement control to one of control of movement within the experiment, and it is increasingly recognized that one very important aspect of the use of visual stimulation is the control of movement in the environment, including posture. Our experiment established the monkey as a model for the study of these systems. Again in humans, damage to the parietal cortex is known to produce certain types of disorientation, and our studies in the monkey open the possibility of dissecting the types of disorientation, particularly those related to full-field visual stimulation.

Proposed Course

In the analysis of the fixation and saccadic systems, a major next step will be the development of a precise model of the saccadic-fixation control system.

This research, to be conducted in this Laboratory in collaboration with Dr. Lance Optican, of LSR, will serve a heuristic function—summarizing the explosive growth of knowledge of the superior colliculus in the past several years. It also will produce a computer-based simulation that will allow tests not only on the visual saccadic-fixation system of the monkey but on that of the human. In experiments on full-field visual stimulation, we will attempt to determine the effect of damage to the cortical areas most likely to be related to the monkey's use of optic flow. These experiments will indicate whether we can identify a specific system that relates optic flow to the behavioral use of this optic flow.

NEI Research Program

Strabismus, Ambylopia, and Visual Processing—Visual Processing and Functional Organization (Structure and Function of Central Visual Pathways)

Publications

Duffy CJ, Wurtz RH: An illusory transformation of optic flow fields. *Vis Res* 33:1481-1490, 1993.

Munoz DP, Wurtz RH: Role of the rostral superior colliculus in active visual fixation and execution of express saccades. *J Neurophysiol* 67:1000-1002, 1992.

Munoz DP, Wurtz RH: Fixation cells in the monkey superior colliculus. I. Characteristics of fixation cells. *J Neurophysiol*, 70:559-575, 1993.

Munoz DP, Wurtz RH: Fixation cells in the monkey superior colliculus. II. Reversible activation and deactivation. *J Neurophysiol*, 70:576-589, 1993.

Wurtz RH, Munoz DP: Role of monkey superior colliculus in control of saccades and fixation. *Cog Neurosci*, in press.

Ophthalmic Genetics and Clinical Services Branch

Report of the Acting Chief, Ophthalmic Genetics and Clinical Services Branch

Muriel I. Kaiser-Kupfer, M.D.

The Ophthalmic Genetics and Clinical Services Branch within the National Eye Institute (NEI) Intramural Research Program has been operational since February 1989. The Branch is organized into four sections: Ophthalmic Genetics, Acting Chief Muriel I. Kaiser-Kupfer, M.D.; Cataract and Corneal Diseases, Chief Manuel B. Datiles, M.D.; Ophthalmic Pathology, Acting Chief W. Gerald Robison, Jr., M.D., Ph.D.; and Clinical Services, Acting Chief Rafael C. Caruso, M.D.

The purpose of the Branch is to conduct clinical and laboratory research on gene expression and molecular interactions important to the eye and to apply clinically relevant research findings to the prevention, diagnosis, and treatment of diseases affecting the eye and visual system. Such disorders include corneal and retinal diseases, cataract, and visual pathway abnormalities.

The Branch is responsible for the essential psychophysical and electrophysiological diagnostic tests of visual function required by clinical intramural research programs of all the Institutes. In addition, it processes ocular clinical biopsy and autopsy materials. The Branch differs from other NEI laboratories engaged in molecular investigations because its emphasis is the translation of appropriate research findings directly to the clinical setting. This Branch is also a point of focus for the trans-National Institutes of Health (NIH) emphasis on research in genetics, more effectively aligning its organizational structure within the Institute's Intramural Research Program. Since beginning its operation, the Branch has shown considerable growth and productivity.

Section on Cataract and Corneal Diseases

The Section on Cataract and Corneal Diseases continued to pursue research on the anterior segment, especially the short- and long-term effects of contact lens wear on the cornea. Analysis of the data may be helpful in understanding the dynamics of contact lens-cornea interaction, the risk to corneal tissues, and how systemic or local ocular disorders may increase the risk of wearing contact lenses. Corneal endothelial morphology is being studied by specular microscopy to compare the endothelial status in patients wearing different types of lenses. The development of automated computer analysis is under way to facilitate data analysis, which currently is performed by hand and is therefore time consuming and laborious.

This Section has been particularly productive in studies using different systems to develop objective and subjective methods of monitoring and documenting opacities in the human lens. Reproducibility studies on objective systems include the use of the Scheimpflug cameras (Zeiss and Oxford) and the retroillumination cameras (Neitz and Oxford). Subjective systems or methods—such as the LOCS II grading system and the effects of cataracts on visual perception, contrast sensitivity, and glare—may be useful in identifying additional parameters. These systems are being used to study the natural history of various cataracts, such as presenile, senile or age-related, steroid-induced, radiation, diabetic, retinitis pigmentosa, gyrate atrophy (GA), and neurofibromatosis 2 (NF2). Genetic linkage studies are under way to pursue the gene(s) of congenital cataracts. Monitoring and documenting human cataract development is a crucial step toward the ultimate testing of several medications that might be helpful in preventing or reversing human cataracts.

Research in cataractogenesis has been hampered by the extreme scarcity of tissue and an abrupt shift in surgical technique, from intracapsular (intact lens) to extracapsular (fragmented lens). Through the collaborative efforts of cataract surgeons and basic researchers, efforts are under way to develop and modify techniques to study materials that become available at surgery and can be well documented clinically. We are carefully documenting the cataract

preoperatively, using clinical and photographic LOCS II grading, as well as Zeiss Scheimpflug and Oxford retroillumination videophotography and image analysis. Cataracts are extracted extracapsularly, followed immediately by implantation of intraocular lenses. Specimens obtained are examined histologically via light and electron microscopy and biochemically via two-dimensional gel electrophoresis (PHAST and LSB systems). Cataractous specimens are compared with normal tissues obtained from eye bank eyes. Abnormal proteins are identified by immunoblotting techniques, as well as by protein sequencing.

It has been demonstrated that with aging there is an acidic shift of proteins and an increased number of polypeptide species in the molecular weight range of the crystallins. These aging changes need to be differentiated from changes occurring in cataract formation.

Investigators in this Section have been in the forefront of recognizing the role of the neural crest in normal and abnormal development of the anterior segment. Studies continue on anterior chamber abnormalities and iridocorneal endothelial syndrome patients.

Section on Ophthalmic Genetics

Studies by the Ophthalmic Genetics Section have emphasized retinal degeneration and ophthalmic involvement in systemic genetic diseases. This Section has been a leader in studying GA of the choroid and retina. The accumulation of natural history data and the work on definition of the genetic abnormalities have been unique. Evidence for biochemical, clinical, and molecular heterogeneity continues to be confirmed. There appear to be many different single-point mutations in the ornithine aminotransferase gene in GA patients. Dietary intervention studies utilizing an arginine deficient diet have been promising, especially in young patients, in whom a delay in the onset of pathologic changes has been demonstrated.

Foveal cone sensitivity (assessed by measurement of increment thresholds) and orientation (estimated by measurement of the Stiles-Crawford effect) were found to be abnormal in a group of patients with GA. These results suggest that foveal cones are altered in their orientation and sensitivity before the encroachment on the foveal area by the atrophic lesions of GA.

Albinism has been associated in animals with an anatomic anomaly of the visual pathways characterized by excessive crossing of the retinogeniculate fibers, with two different modes of geniculocortical projection. In humans, indirect evidence of the same anomaly is demonstrated by asymmetry in visually evoked potentials (VEPs) elicited by pattern-reversal stimulation. Recent studies using appearing-disappearing patterns claimed VEP asymmetry to be diagnostic and proposed a uniform type of asymmetry. We used the same recording conditions to determine the diagnostic value of VEP in albinism and to attempt to correlate the VEP results with clinical features.

This study shows that there are two different patterns of VEP asymmetry in albinism, which may be explained by differences in reorganization of the geniculocortical pathway. VEP asymmetry occurs frequently but may not be constant in this condition. However, its value is decreased in some cases in which the low amplitude of the responses makes interpretation difficult. Furthermore, there is no correlation of the type of asymmetry with any other feature of albinism.

Collaboration with the Interinstitute Genetics Program has continued, with active participation by the Genetics Clinic. During the past year, we have seen approximately 200 individuals representing approximately 60 different disease categories. Because of the high frequency of ocular involvement in these cases, almost all of these patients were evaluated by the Ophthalmic Genetics staff.

NF2, otherwise known as bilateral acoustic neuroma, is inherited as an autosomal dominant disorder. Multiple members of several large pedigrees, as well as a large number of unrelated families, have been studied in collaboration with Dr. Dilys Parry (National Cancer Institute). An important original observation was the striking frequency of posterior capsular cataract in patients with NF2 (80-85%). In addition, 30% of the patients have shown associated cortical cataracts. These findings are helpful in establishing a diagnosis of NF2 in at-risk patients. The etiology of the cataract is unclear; however, it is interesting that the gene locus for bilateral acoustic neuromas is on chromosome 22, as is the gene for βB-crystalline. Combined pigment

epithelial and retinal hamartomas appear to be another ocular marker for some patients with severe NF2.

GA is a condition amenable to gene therapy; preliminary laboratory studies are under way toward that goal. Usher's syndrome, congenital deafness, and retinitis pigmentosa patients are being studied; molecular techniques are used to map the gene and to identify the responsible mutation.

Finally, the results from the continuing double-masked, controlled clinical trial of topical cysteamine patients with nephropathic cystinosis are exciting. Since confirming the usefulness of 0.5% cysteamine eye drops in the young patients, we expanded our study to include older patients, with similarly striking results. Particularly important is the fact that these patients have shown dramatic relief from their ocular symptoms, with a decrease in crystals in the treated eye and a significant improvement in their quality of life.

Section on Clinical Services

The Clinical Services Section has been active in characterizing psychophysical and electrophysiological findings in patients with diseases that affect the eye and the visual system. Continued documentation by noninvasive techniques has shown that more and more refined and accurate classification of diseases is possible. Psychophysical and electrophysiological information is particularly helpful in understanding the pathogenesis of disease, as well as being available for use as a marker in various treatment modalities.

The results of VEP studies showed that two waveforms, which frequently show the same surface-positive polarity but are generated by stimulation of each hemifield, combine to generate peaks of the full-field VEP.

Our results indicate that the sum of the asymmetrical contributions of both eyes (either hemisphere of each) is responsible for the symmetrical VEP elicited by binocular stimulation with a full-field stimulus. An asymmetrical, full-field VEP may occur in normal subjects and does not imply an abnormality in the visual pathways.

Studies of dark adaption (DA) in patients with retinal dystrophies indicated that a complete evaluation of DA should include, in addition to measurement of DA, the time constant of adaptation, which provides information about the rate at which this final threshold is reached. The time constant serves as a clinically relevant parameter in both the diagnosis of retinopathies and the followup of individual patients over time.

Section on Ophthalmic Pathology

The Section on Ophthalmic Pathology has provided technical support services to investigators involved in clinical and basic research as well as to those performing routine pathology. Careful monitoring of the volume of material handled shows a steady increase in processing by the Laboratory, with excellent results. Considerable savings to the Institute have resulted from the elimination of costly contract services.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00187-10 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Effects of Corneal Contact Lenses on the Cornea

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Manuel B. Datiles	M.D.	Medical Officer	OGCSB, NEI
Others:	Gregory P. Kracher	O.D.	Expert	OGCSB, NEI
	Lessie McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Louella Lopez	M.D.	Visiting Associate	OGCSB, NEI
	Doretha Leftwood	B.A.	Computer Specialist	OGCSB, NEI
	Anup Mahurkar	B.S.	Visiting Associate	OGCSB, NEI

COOPERATING UNITS *(if any)*

Image Processing and Analysis Laboratory, Division of Computer Research and Technology, NIH (Mark Vivino, B.S.)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Cataract and Corneal Diseases

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.575	0.450	0.125

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This investigation of the short-term as well as the long-term effects of contact lens wear on the cornea includes specular microscopy studies of changes in corneal curvature, corneal epithelial morphology, and corneal endothelial cell morphology. Analysis of the data obtained will help us understand the dynamics involved in the interaction between a contact lens and the cornea, the risk to corneal tissues, and how a systemic or local disorder may increase these risks. In addition, we are studying the differences in corneal endothelial status of wearers of soft compared with hard contact lenses.

Animal models showing corneal endothelial abnormalities similar to those in long-term contact lens wearers also are being explored in diabetic and galactosemic animal models. Treatment with aldose reductase inhibitors helps prevent these corneal abnormalities.

PHS 6040 (Rev. 5/92)

Project Description

Clinical Protocol Number

84 EI-133

Objectives

The objective of this project is to investigate the effects of contact lens wear on corneal tissues, including the study of factors that increase or decrease the potential risk of injury to corneal tissues by contact lens wear.

Methods

We used each patient's complete history, ophthalmologic examination, photography, keratotometry, pachymetry, and specular microscopy of the corneal endothelium.

Major Findings

We have found that diabetes may increase the risk of complications from contact lenses in the first 6 months of wear. In addition, we have found changes in the corneal endothelium after long-term wear of contact lenses. These changes include polymegathism and pleomorphism. Furthermore, 2 years after some of our patients discontinued wearing contact lenses, we found a trend toward recovery but no statistically significant change.

In addition, we found that diabetic and galactosemic animals have these endothelial abnormalities and that treatment with aldose reductase inhibitors prevented these abnormalities.

Significance to Biomedical Research and the Program of the Institute

Contact lenses are commonly used for correction of errors of refraction as well as for therapy. However, our knowledge of the interaction of contact lenses with the cornea and tears is limited. In addition, risks associated with wearing contact lenses are poorly understood. Understanding the interaction between contact lenses and corneal tissues will allow us to determine why some patients cannot wear contact lenses and provide methods to avoid some of the complications associated with contact lens wear.

Proposed Course

The following studies are in progress or proposed for next year: (1) continued examination of patients who have worn hard contact lenses and have now shifted to gas-permeable or soft contact lenses, (2) recruitment of patients who plan to discontinue contact lens wear or to shift from one type of contact lens to another; and (3) development of automated computer analysis of all types to facilitate the analysis of data.

NEI Research Program

Corneal Diseases—Corneal Edema, Endothelial Dysfunction, Dystrophies and Inherited Disease

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00188-10 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Documentation and Monitoring of Opacities in the Human Lens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Manuel B. Datiles	M.D.	Medical Officer	OGCSB, NEI
Others:	Benjamin Magno	M.D.	Visiting Associate	OGCSB, NEI
	Maria Susan Lasa	M.D.	Visiting Associate	OGCSB, NEI
	Louella Lopez	M.D.	Visiting Associate	OGCSB, NEI
	Anup Mahurkar	B.S.	Visiting Associate	OGCSB, NEI
	Doretha Leftwood	B.A.	Computer Specialist	OGCSB, NEI
	Joan Lee		Clinic Coordinator	OGCSB, NEI

COOPERATING UNITS *(if any)*

Image Processing and Analysis Laboratory, Division of Computer Research and Technology (DCRT), NIH (Benes Trus, Ph.D.; Mark Vivino, B.S.); Biomedical, Engineering and Instrumentatioo Branch, DCRT, NIH (Michael Unser, Ph.D.); Epidemiology Branch, NEI, NIH (Michael Unser, Ph.D.); Clinical and Diagnostic Trials Sectioo, National Cancer Institute, NIH (Sylvan Green, M.D.); Nuclear Medicine, Clinical Center, NIH (Joseph Frank, M.D.)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Cataract and Corneal Diseases

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.725	2.30	1.425

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project uses different systems to develop objective and subjective methods to monitor and document opacities in the human lens. We are actively recruiting patients with and without cataracts for reproducibility studies on the objective systems—the Scheimpflug (Zeiss and Oxford) and retroillumination (Neitz and Oxford) cameras. Our study of subjective systems or methods, such as the LOCS II grading system, and the effects of cataracts on visual perception, contrast sensitivity, and glare may be useful in identifying additional parameters for monitoring cataract presence, progression, or regression. We are now using these systems to study the natural history of various cataracts, such as presenile, senile, or age-related, steroid-induced, radiation, diabetic, retinitis pigmentosa, gyrate atrophy, and neurofibromatosis II cataracts. This study will prepare the way for eventual clinical trials of anticataract drugs.

Genetic linkage studies under way are pursuing the gene(s) of congenital cataract.

Project Description

Additional Personnel

Malina A. Drews-Bankiewicz	M.D.	Visiting Fellow, OGCSB, NEI
Yvonne Duglas-Tabor	B.S.	Biologist, OGCSB, NEI
Marvin Podgor	M.D.	Epidemiologist, NEI
Robert Sperduto	M.D.	Chief, Epidemiology Section, NEI
Laura Wozencraft	B.S.	Genetic Counselor, OGCSB, NEI
Mark H. Scott	M.D.	Senior Staff Fellow, OGCSB, NEI
J. Fielding Hejtmancik	M.D., Ph.D.	Staff Scientist LMOD, NEI

Clinical Protocol Number

84-EI-132

Objectives

The objective of this project is to formulate means of documenting cataract formation and progression. This is an important step prior to undertaking clinical trials of drugs purported to prevent cataract and cataract progression. Family studies are involved in looking for the gene for congenital cataract via linkage studies.

Methods

Complete ophthalmologic examination, including contrast sensitivity, glare testing, and potential acuity testing, are performed for each person in the study. Techniques used to measure and evaluate cataracts include Scheimpflug photography, retroillumination photography, specular microscopy, and laser light-scanning spectroscopy.

Major Findings

We have found that clinical grading of cataracts using the LOCS II system is a means of quantitatively detecting the progression of age-related cataracts within 1 year. In addition, we found that in various types of cataracts, glare and contrast sensitivity testing show abnormal results only in the severe or more advanced grades. The only exception was in posterior subcapsular cataracts, which showed an abnormality in contrast and glare sensitivity in the early stages, based on LOCS II grading. In a study of pure nuclear cataracts, we found a significant correlation between lens nuclear density (assessed by either LOCS II grading or Scheimpflug photography) and contrast sensitivity loss of intermediate and high spatial frequencies.

In our continued development of objective, semiautomated methods of detecting and following cataracts, we now are able to perform quickly densitometry of Scheimpflug nuclear cataract images and compare the results with previous images to detect significant changes, which are expressed in optical density units. For posterior subcapsular and cortical cataract, we also have developed a semiautomated method of quantitating the cataracts in square millimeters using retroillumination photography.

Significance to Biomedical Research and the Program of the Institute

The monitoring and documenting of human cataract progression is a crucial step toward the ultimate testing of several medications believed capable of preventing or reversing human cataracts. This step is also important in categorizing types of cataracts in various parts of the world and correlating them with physical and genetic factors in specific geographic regions.

Subjective methods of determining visual function are also important to determine the degree of handicap that cataract patients have in coping with daily activities. Since in our studies none of the subjective methods could demonstrate subjective experiences in association with early cataracts, research is needed to develop more sensitive techniques.

Proposed Course

We will continue the study and development of subjective and objective methods of documenting and monitoring human cataracts. We will pursue the improvement and automation of the present system of lens photography (e.g., Scheimpflug, retroillumination, and laser-light spectroscopy), as well as exploration of possible applications of new technological advances. Appropriate population groups for study will be identified.

NEI Research Program

Cataracts—Epidemiology of Cataract

Publications

Datiles M: Clinical evaluation of cataracts, in Tasman W, Jaeger E (eds): *Duane's Clinical Ophthalmology.* Philadelphia, Lippincott Co., 1992, Vol. I 73B, pp 1-16.

Datiles M, Magno B, Leftwood D, Friedlin V, Vivino M: Longitudinal study of age related nuclear cataracts using the NEI Scheimpflug Imaging System. *Investig Ophthalmol Vis Sci* 34:4a, 943, 1993.

Fox PC, Datiles M, Atkinson JC, Scott J, Fletcher D, Valdez IH, et al: Prednisone and piroxicam for treatment of primary Sjogren's syndrome. *Clin Exp Rheumatol* 11:149-156, 1993.

Genhart M, Kelly K, Coursey R, Datiles M, Rosenthal N: Effects of bright light on mood in normal elderly women. *Psychiatry Res* 47:87-97, 1993.

Kashima K, Trus BL, Unser M, Datiles MB, Edwards PA, Sibug M: Aging studies on normal volunteer lenses using the Scheimpflug slit lamp camera. *Invest Ophthalmol Vis Sci* 34:263-269, 1993.

Kashima K, Unser M, Datiles MB, Trus BL, Edwards PA: Minimum views required to characterize cataracts when using the Scheimpflug camera. *Graef Arch Ophthalmol,* 231: 687-691, 1993.

Lasa S, Datiles MB: Longitudinal study of cortical cataracts using retroillumination photographs. *Investig Ophthalmol Vis Sci* 34:4a, 943, 1993.

Lasa S, Podgor M, Datiles M, Magno B: Glare sensitivity in early cataracts. *Br J Ophthalmol* 77:489-491, 1993.

Lopez ML, Datiles M: Longitudinal study of age related posterior subcapsular opacities using retroillumination photographs. *Investig Opthalmol Vis Sci* 34:4a, 943, 1993.

Magno B, Datiles M, Friedlin V: Reproducibility of the NEI Scheimpflug Cataract Imaging System. *Investig Ophthalmol Vis Sci* 34:4a, 943, 1993.

Magno BL, Datiles MB, Lasa SM: Senile cataract progression studies using the Lens Opacity Classification System. *Invest Ophthalmol Vis Sci* 34:2138-2141, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00212-08 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Use of Human Lens Material for Determining Possible Causes of Cataracts

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Manuel B. Datiles	M.D.	Medical Officer	OGCSB, NEI
Others:	Maria Susan Lasa	M.D.	Visiting Associate	OGCSB, NEI
	Benjamin Magno	M.D.	Visiting Associate	OGCSB, NEI
	Yvonne Tabor	B.S.	Biological Technician	OGCSB, NEI
	Louella Lopez	M.D.	Visiting Associate	OGCSB, NEI
	Pushpa Sran	M.D.	Medical Officer	OGCSB, NEI

COOPERATING UNITS *(if any)*

Laboratory of Mechanisms of Ocular Diseases, NEI (Donita Garland, Ph.D.); Laboratory of Immunology, NEI (Miguel Burnier, Jr., M.D.)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Cataract and Corneal Diseases

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.05	1.75	1.30

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

There is an extreme scarcity of properly documented and classified human cataract material because of an abrupt shift of cataract surgical technique from intracapsular (intact lens) to extracapsular (fragmented lens) with the advent of the use of intraocular lenses. We are exploring ways by which fragmented lens materials can be maximally used in cataract basic research through close collaboration with cataract surgeons and basic researchers and through modification of techniques by both groups.

We are now carefully documenting the cataracts preoperatively, using clinical and photographic LOCS II grading and Scheimpflug (Zeiss and Oxford) retroillumination (Oxford) video photography and image analysis. Cataracts are extracted extracapsularly with implantation of an intraocular lens. Specimens obtained are examined histologically, using light and electron microscopy, and biochemically, using two-dimensional gel electrophoresis (PHAST and LSB systems). Cataractous specimens are compared with normal tissues obtained from eye bank eyes. Abnormal proteins are identified using immunoblotting techniques as well as protein sequencing.

Project Description

Additional Personnel

Samuel Zigler	Ph.D.	Head, Section on Cataracts, LMOD, NEI

Clinical Protocol Number

84-EI-194

Objectives

In light of the present and future scarcity of intact human cataracts for basic research, this study was designed to develop methods in which fragmented lens materials can be maximally used for biochemical and genetic research.

Methods

We examined patients who have different forms of cataracts and documented their cataracts, as described in project Z01 EY 00188-10, Documentation and Monitoring of Opacities in the Human Lens. After extracapsular cataract extraction, fragmented lens materials—which include the anterior lens capsule, lens nucleus, and aspirated lens cortical material—undergo protein and biochemical analyses. Some of the specimens are frozen for future genetic studies. We also have undertaken studies to determine the visual results of current techniques of cataract surgery and how to modify and improve them further.

Major Findings

We have found that a successful collaboration requires a close professional relationship between the clinician-surgeon and the basic researcher. Although the two professionals have different immediate priorities (one being patient care; the other, adequacy of tissue samples for laboratory studies), the ultimate goal of alleviating human suffering is the same.

The two-dimensional gel electrophoresis technique may be extremely useful in determining lens protein changes using very small amounts of tissue (300 mcg). Aging results in acidic shifting of proteins, an increased number of polypeptide species in the molecular weight range of the crystallins, and covalent cross-linking of crystallins—changes that need to be differentiated from changes occurring in cataract formation. We also have found that lens material aspirated through the irrigator-aspirator or phakoemulsifier lose some crystallins; optimum samples are those we obtain separately, thus avoiding oxidation.

Significance to Biomedical Research and the Program of the Institute

A severe setback is being dealt many cataract projects because of the lack of human cataract material available for basic research studies. While the current technique, which involves fragmenting the cataract during extraction, is extremely successful and effective, there is no foreseeable change back to utilization of the intracapsular method (i.e., removal of the lens in toto). Hence, it is imperative that we modify our basic research methodology to adapt to the use of fragmented lens materials in order to continue basic lens research projects that deal with human materials.

Proposed Course

We will continue to pursue the development of the use of fragmented lens material in basic research experiments. Using two-dimensional gel electrophoresis, we will study ways in which surgeons can modify their surgical techniques without compromising patient care while providing scientists with critical lens tissue for basic research. In addition, we will investigate ways in which scientists can work with surgeons in handling lens materials to maximize the quality of specimens for basic research.

NEI Research Program

Cataract—Treatment of Cataract and Correction of Aphakia

Publications

Datiles M, Kinoshita J: Pathogenesis of cataracts, in Tasman W, Jaeger E (eds): *Duane's Clinical Ophthalmology.* Philadelphia, Lippincott Co., 1991, 72B, pp 1-14.

Garland D, Tabor Y, Zigler JS, Datiles MB: Protein analysis of lens cortical aspirates obtained at surgery. *Investig Ophthalmol Vis Sci* 34:4a, 1335, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00281-01 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Addendum to Use of Human Lens Material for Determining Possible Causes of Cataracts

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	J. Fielding Hejtmancik	M.D., Ph.D.		LMOD, NEI
	Mark H. Scott	M.D.	Senior Staff Fellow	OGCSB, NEI

COOPERATING UNITS *(if any)*

Marshall Parks, M.D. (Private Practice), Washington, DC

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Cataract and Corneal Diseases

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.325	1.325	0.0

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [x] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Although the etiologies of some secondary cataracts are becoming better understood and certain animal models show promise for elucidating the relationships between lens crystalline and hereditary cataract, little is known about the causes of congenital cataracts in humans. To date, the classification of different congenital cataracts has been cumbersome and imperfect. A better understanding of cataractogenesis will come through an understanding of the molecular components of the lens of the eye and the ways in which lesions of these components are manifested, structurally and functionally, as opacities of the lens. Animal studies have suggested that alterations in lens crystalline can cause hereditary cataracts, making them reasonable candidate genes for causing hereditary cataracts in humans. In addition, it is apparent that hereditary lesions that mimic or contribute additively to environmental stress known to cause cataracts might be candidate genes for causing hereditary cataracts. The work in this project is designed to concentrate specifically on congenital and hereditary cataracts and to take full advantage of molecular technology developed for linkage analysis. Studies performed on informative families will include collecting blood specimens from available family members and, when possible, analyzing lens material from patients who undergo cataract surgery.

Project Description

Clinical Protocol Number

84 EI-194A

Objectives

The objective of this study is to do linkage analysis using molecular genetic techniques in families with congenital hereditary cataracts.

Methods

Linkage analysis (i.e., gene mapping) will be carried out by following the inheritance of genetic markers in families with hereditary cataracts. In informative families, blood specimens will be obtained and analyzed for gene marker linkage analysis.

Major Findings

Under way at present is the enrollment of families with congenital cataracts. A large family with known autosomal dominant congenital cataract has been analyzed. This analysis is the first to provide evidence of intraocular phenotypic heterogeneity in a family with autosomal dominant congenital cataract. Studies for markers for gene analysis are under way.

Significance to Biomedical Research and the Program of the Institute

By studying patients with congenital inherited cataract, it may be possible to find the specific gene responsible for the development of congenital cataracts.

Proposed Course

More families who have congenital cataract will be recruited, and linkage analysis will be performed on these families.

NEI Research Program

Cataract—Treatment of Cataract and Correction of Aphakia

Publications

Hejtmancik JF, Kaiser-Kupfer MI, Piatigorsky J: Molecular biology and inherited disorders of the eye lens, in Scriver CR, Beaudet AL, Sly WS, Valle D (eds): *Metabolics Basics of Inherited Disease.* McGraw-Hill Inc., submitted.

Scott MH, Hejtmancik JF, Wozencraft LA, Reuter LM, Parks MM, Kaiser-Kupfer MI: Autosomal dominant congenital cataract: Interocular phenotypic heterogeneity. *Ophthalmology*, submitted.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00084-15 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Anterior Chamber Anomalies Associated With Glaucoma or Ocular Hypertension

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Neme, title, laboratory, and institute affiliation)*

PI:	Carl Kupfer	M.D.	Director	NEI
Others:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
	Lessie McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Manuel B. Datiles	M.D.	Medical Officer	OGCSB, NEI
	Maria Susan Lasa	M.D.	Visiting Associate	OGCSB, NEI
	Benjamin V. Magno	M.D.	Visiting Associate	OGCSB, NEI
	Louella Lopez	M.D.	Visiting Associate	OGCSB, NEI

COOPERATING UNITS *(if eny)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Cataract and Corneal Diseases

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	0.7	0.3

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

Recent embryological research has indicated the role of the neural crest in contributing to all connective tissues anterior to the lens epithelium. Therefore, the group of developmental anomalies of the anterior chamber with glaucoma or ocular hypertension is being reviewed.

Project Description

Clinical Protocol Number

77-EI-119

Objectives

The object of this study is to determine whether congenital or developmental anomalies of the anterior chamber are related to faulty migration or terminal differentiation of neural crest tissue.

Methods

Patients of all ages with congenital or developmental anomalies of the anterior chamber are being examined to determine involvement of cornea, trabecular meshwork, iris stroma, lens, and ciliary body. When intractable glaucoma cannot be controlled with medication, surgery is performed and the specimens are examined histologically. When the lenses become cataractous, cataract extractions are performed and the lens epithelium is grown in tissue culture. When the cornea is opaque and corneal transplantation is indicated, that procedure is performed and the corneal specimen is examined histologically.

Major Findings

It appears that, in this group of anomalies of anterior chamber development, there are pathological changes in one or several tissues derived from neural crest. These changes include corneal stroma, corneal endothelium, anterior iris stroma, Descemet's membrane, and trabecular meshwork endothelium.

We recently performed trabeculectomies on patients with the irido-corneal-endothelial syndrome. Histopathologically, we found the presence of a membrane covering the trabecular meshwork. That membrane may have caused the peripheral anterior synechias and glaucoma.

Significance to Biomedical Research and the Program of the Institute

A better understanding of the pathogenesis of this glaucoma may help by improving diagnosis and treatment. The presence of this membrane may explain the glaucoma's progressive nature and suggest possible surgical or laser treatments as a way to control or prevent the progression of the disease.

Proposed Course

Patients with other anomalies of the anterior chamber, including congenital cataracts, will be examined for abnormalities in tissue derived from neural crests. We will continue to study cases of congenital corneal disorders to uncover the cause and to determine treatment choices for these cases.

NEI Research Program

Glaucoma—Other Glaucoma (Developmental, Congenital, and Infantile Glaucoma)

Publications

Kupfer C, Chan C-C, Burnier M Jr, Kaiser-Kupfer MI: Histopathology of the ICE syndrome. *Trans Am Ophthalmol Soc* 90:149-160, 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00123-13 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Clinical Psychophysics of the Visual System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
Others:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
	Malina Drews-Bankiewicz	M.D.	Visiting Associate	OGCSB, NEI
	Doris J. Collie	A.A.	Ophthalmic Technician	OGCSB, NEI
	Patricia A. Mercer	M.P.A.	Ophthalmic Technician	OGCSB, NEI
	Leanne M. Reuter	B.S.	Ophthalmic Technician	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Eye Services Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.49	0.41	1.08

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The visual function of patients with ocular diseases or lesions in the visual pathways and of normal subjects is measured using psychophysical techniques. The data are correlated with those obtained with electrophysiological tests of visual function. The results contribute to the diagnosis of ocular and neural disorders that affect vision and are needed to characterize their nature and evolution. They are also valuable in the assessment of how different forms of treatment affect the outcome of these diseases.

Project Description

Clinical Protocol Number

93-EI-0131

Objectives

The aims of this project are (1) to apply and develop psychophysical techniques for the study of vision in the clinical setting, (2) to characterize the human visual system's normal function, and (3) to analyze the patterns of its alteration in ocular diseases and lesions of the visual pathways.

Methods

Several psychophysical techniques are employed as discussed below:

Perimetry.—Visual fields are explored with kinetic quantitative perimetry and static quantitative perimetry.

Color vision.—Central color vision is estimated using HRR pseudoisochromatic plates, Ishihara pseudoisochromatic plates, Farnsworth's Tritan plate, Farnsworth-Munsell D-15 panel, Lanthony's desaturated D-15 panel, Farnsworth-Munsell 100 hue test, and the Nagel anomaloscope.

Adaptometry.—Dark-adapted rod and cone thresholds are measured with a modified Goldmann-Weekers adaptometer.

Spatial vision.—The spatial contrast sensitivity function is determined using sinusoidal luminance gratings. A two-alternative temporal forced-choice technique is used for a criterion-free judgment of threshold visibility.

Luminance and chromatic increment thresholds.—These are measured with a two-channel Maxwellian view instrument. This instrument also is used to assess retinal receptor orientation by measuring the Stiles-Crawford effect (SCE of the first kind).

Major Findings

The diagnostic value of the time constant of the dark adaptation (DA) function as an estimator for the evaluation of visual function was studied in patients with retinal degenerations. Seven groups (70 subjects) were included in this study: patients with retinitis pigmentosa (RP) (12), gyrate atrophy (GA) (15), cone dystrophy (11), juvenile macular dystrophy (9), fundus flavimaculatus (FF) (33), other retinal degenerations (6), and normal subjects (14). The results obtained on one eye chosen at random in each subject were analyzed. Measurements were made with a modified Goldmann-Weekers adaptometer. The subjects were initially light adapted with a 2,550 cd/m^2 background for 5 minutes. DA functions were obtained by measuring the change in absolute threshold with time (using von Bekesy tracking) for 30 minutes. The following model (Pugh, 1975) was used to fit each of the two limbs of the DA function:

$$\text{Threshold} = a + b \cdot \overline{e}^{\frac{\text{time}}{c}}$$

In this model, "a" is the final DA threshold (in dB), "b" is the magnitude of DA (in dB), and "c" is the time constant of DA (in minutes). Analysis of variance was used to examine differences between the means of each parameter among the study groups. Statistically significant differences in final threshold ("a") and time constant ("c") were seen in both the cone- and rod-mediated limbs of the DA function. Abnormally high time constants in the cone-mediated limb were observed in RP and GA patients. FF patients who had a normal final threshold had abnormal elevation in the time constant of rod-mediated limbs. The usual DA estimator is the final absolute threshold. These findings suggest that a complete evaluation of DA should include, in addition to a measurement of the final threshold, the time constant of adaptation, which provides information about the rate at which this final threshold is reached. The time constant serves as a clinically relevant parameter in both the diagnosis of retinopathies and the followup of individual patients over time.

Significance to Biomedical Research and the Program of the Institute

Psychophysical techniques are noninvasive methods useful in the diagnosis and management of ocular diseases and visual pathway lesions. In addition to the application of validated tests, the development of new techniques contributes to the elucidation of the pathophysiological mechanisms of visual disorders.

Proposed Course

Clinical psychophysical studies of visual function in diseases of the eye and visual pathways will be

continued. We will introduce modifications that are expected to enhance the diagnostic value of the techniques described.

NEI Research Program

Retinal and Choroidal Diseases—Noninvasive Techniques in the Study of Retinal Disorders

Strabismus Amblyopia, and Visual Processing—Visual Processing and Amblyopia

Publications

Drews-Bankiewicz MA, Caruso RC, Kaiser-Kupfer MI: The clinical relevance of the time course of dark adaptation in retinal degenerative diseases. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1077, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 EY 00144-12 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Clinical Electrophysiology of the Visual System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
Others:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
	Malina Drews-Bankiewicz	M.D.	Visiting Associate	OGCSB, NEI
	Patricia A. Mercer	M.P.A.	Ophthalmic Technician	OGCSB, NEI
	Doris J. Collie	A.A.	Ophthalmic Technician	OGCSB, NEI
	Leanne M. Reuter	B.S.	Ophthalmic Technician	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Eye Services Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.41	0.41	1.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [X] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The visual function of patients with ocular diseases or lesions in the visual pathways and of normal subjects is measured objectively using electrophysiological techniques. The data are correlated with those obtained with psychophysical tests of visual function. The results contribute to the diagnosis of ocular and neural disorders that affect vision and are needed to characterize their nature and evolution. They are also valuable in the assessment of the effects of different forms of treatment on the outcome of these diseases.

Project Description

Clinical Protocol Numbers

91-EI-26
82-EI-55

Objectives

The aims of this project are (1) to apply and develop electrophysiological techniques for the study of visual function in the clinical setting, (2) to characterize the normal electrical activity of the human visual system, and (3) to analyze the patterns of its alteration in ocular diseases and lesions of the visual pathways.

Methods

The electrophysiological techniques employed involve recording potentials generated by the retinal pigment epithelium (electrooculogram), the neural retina (electroretinogram), and the central visual pathways (visually evoked potentials [VEPs]). These potentials are elicited by unstructured stimuli (Ganzfeld full-field or focal stimulation) and spatially structured stimuli (sinusoidal gratings or checkerboard patterns).

Major Findings

The transient pattern reversal VEP elicited by stimulation of the central visual field is characterized by a typical negative–positive–negative (NPN) waveform. Previous reports using hemifield stimulation suggest that the peaks similar to those elicited by full-field stimulation arise primarily from the ipsilateral electrode sites. In this study, we investigated the contributions of both ipsilateral and contralateral electrode sites to the origin of the full-field VEP peaks. Fifty normal human subjects were studied.

VEPs elicited by the contrast reversal of a 4 cycles/degree sinusoidal grating were recorded over five electrode sites 5 cm above the inion (midline) and 5 cm (occipital) and 10 cm (temporal) to the left and right of the midline. VEPs were elicited by binocular and monocular stimulation of the full (26° × 20°) field and of the left and right (13° × 20°) hemifields.

VEPs elicited by stimulation of a hemifield have been described as consisting of NPN waveforms over the ipsilateral electrodes and variable low-amplitude responses of opposite polarity (i.e., surface-negative) over the contralateral electrodes. However, in our study, hemifield stimuli elicited asymmetrical surface-positive waveforms on both sides of the midline in most cases (92%). The amplitude of these waveforms was larger over the electrodes ipsilateral to the stimulated hemifield ("paradoxical" lateralization). Their main positive peak occurred earlier over the electrodes contralateral to the stimulated hemifield. The peaks of the full field VEP at each electrode site were generated by the algebraic sum of the peaks of the hemifield VEPs. This sum of two hemifield responses closely matched the full-field VEP, with negative peaks being frequently generated by a single hemisphere. A summation of two waveforms, which very frequently show the same surface-positive polarity and are generated by stimulation of each hemifield, generates the peaks of the full-field VEP.

The symmetry of pattern-reversal VEPs recorded to the left and right of the midline has been proposed as a valuable estimator of the functional integrity of the visual pathway. We explored the variability of VEP symmetry and analyzed the contribution of both eyes and both hemispheres to this symmetrical response. Fifty normal human subjects underwent an ophthalmologic examination, including a 30° visual field test (static perimetry). Transient VEPs elicited by the contrast reversal of a 4 cycles/degree sinusoidal grating were recorded over five electrode sites 5 cm above the inion. VEPs were elicited by binocular and monocular stimulation of the full (26° × 20°) field and of the left and right (13° × 20°) hemifields.

Full field stimulus.—After binocular stimulation, mean VEP amplitudes were symmetrical about the midline. However, asymmetries in the amplitude of VEPs were seen in individual subjects. Therefore, 95% tolerance intervals about the mean amplitude differences (left lead minus right lead) are large (−11.42 to 9.55 μV for the occipital sites and −4.80 to 5.18 μV for the temporal sites). Monocular stimulation of either OD or OS generated larger mean amplitudes over the ipsilateral electrodes. This voltage difference was not large but significant ($p < 0.001$).

Hemifield stimulus.—VEPs elicited by stimulation of a hemifield were asymmetrical. They consistently showed larger amplitudes over the ipsilateral electrodes (paradoxical lateralization). The sum of the two asymmetrical hemifield responses was symmetri-

cal and closely matched the full-field VEP. Our results indicate that the sum of the asymmetrical contribution of either hemisphere and either eye are responsible for the symmetrical VEP elicited by binocular stimulation with a full-field stimulus. An asymmetrical full-field VEP may be seen in normal subjects and does not imply an abnormality in the visual pathways.

Significance to Biomedical Research and the Program of the Institute

Electrophysiological techniques are noninvasive methods useful in the diagnosis and management of ocular diseases and visual pathway lesions. In addition to validating tests, the new techniques developed contribute to the elucidation of the pathophysiological mechanisms of visual disorders.

Proposed Course

We will continue clinical electrophysiological studies of visual function in diseases of the eye and visual pathways, introducing modifications expected to enhance the diagnostic value of the techniques described.

NEI Research Program

Retinal and Choroidal Diseases—Noninvasive Techniques in the Study of Retinal Disorders

Strabismus, Amblyopia, and Visual Processing—Visual Processing and Amblyopia

Publications

Caruso RC, Reuter LM, Muller SF, Drews-Bankiewicz MA, Kaiser-Kupfer MI: Origin of the peaks of the human transient pattern reversal visual evoked potential. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1276, 1993.

Reuter LM, Caruso RC, Muller SF, Drews-Bankiewicz MA, Bouzas EA, Kaiser-Kupfer MI: The pattern reversal visual evoked potentials: Symmetrical or asymmetrical. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1276, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00257-05 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visual Function Diagnosis Service

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
Others:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
	Tracy T. Nolan	M.A.	Health Technician	OGCSB, NEI
	Dessie Koutsandreas	C.O.A.	Ophthalmic Technician	OGCSB, NEI
	Anne Randall	C.O.M.T.	Ophthalmic Technician	OGCSB, NEI
	Linda Goodman	C.O.T.	Ophthalmic Technician	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Eye Services Section

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.81	0.15	6.66

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This general service project provides diagnostic support for all research protocols conducted by the Clinical Sections of the NEI and other Institutes that require an assessment of visual function. Psychophysical and electrophysiological techniques are used to detect and quantify visual loss due to disorders of the ocular media, uvea, retina, optic nerve, and central visual pathways.

Project Description

Additional Personnel

R. Patrick McDaniel	Ophthalmic Technician, OGCSB, NEI
Antoinette LaReau	Ophthalmic Technician, OGCSB, NEI
Sueli Müller	Special Volunteer, OGCSB, NEI

Objectives

The aim of this project is to provide accurate measurements of visual function for the differential diagnosis of visual loss. The first step in this process is detection of a visual deficit (i.e., determining whether visual loss has occurred). The second step is the quantification of a detected deficit. The third is an analysis of the characteristics of the visual deficit to determine the site of the lesion responsible for this symptom (topographic diagnosis). The final step is correlation with other clinical findings to ascribe the visual deficit to a given pathological process.

Methods

The psychophysical techniques employed include commercially available and laboratory-developed techniques for the measurement of visual acuity, visual fields, color vision, dark adaptation, spatial contrast sensitivity, and glare disability.

The electrophysiological techniques used include recording potentials generated by the retinal pigment epithelium (electrooculogram), the neural retina (electroretinogram), and the central visual pathways (visually evoked potentials).

Major Findings

During the period October 1, 1992, through September 30, 1993, we performed the following tests:

Kinetic perimetry	312
Static perimetry	413
Screening perimetry	102
Manifest refraction	1,313
Color vision tests	193
Adaptometry	45
Contrast sensitivity tests	18
Glare disability tests	5
Ganzfeld electroretinography	181
Focal electroretinography	50
Electrooculography	102
Visually evoked potentials	117

This represents an increase of 57% over the tests performed during the same period in Fiscal Year 1992.

Significance to Biomedical Research and the Program of the Institute

This project provides all tests of visual function for patients who visit the NEI Eye Clinic. In the majority of ophthalmologic diseases, visual loss is the most meaningful finding. In most clinical research protocols involving diseases of the eye and visual pathways, visual deficit is used as an indicator of the progress of a disease or the effect of a treatment. Therefore, sensitive and accurate measurements of visual function are essential for these clinical research projects.

Proposed Course

The provision of clinical electrophysiological and psychophysical tests of visual function for patients with diseases of the eye and visual pathways will be continued. We will introduce modifications that are expected to enhance the diagnostic value of the techniques described.

NEI Research Program

Retinal and Choroidal Diseases—Noninvasive Techniques in the Study of Retinal Disorders

Strabismus, Amblyopia and Visual Processing—Visual Processing and Amblyopia

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00011-19 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pigment Dispersion With and Without Glaucoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	Lessie McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Mark H. Scott	M.D.	Senior Staff Fellow	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.25	0.15	0.1

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [x] (a1) Minors
  - [x] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to determine the risks of patients with pigment dispersion syndrome for glaucoma. Comparisons of patients with and without glaucoma are made on the basis of diagnostic tests, genetic screening, and aqueous humor dynamics. The data acquired may enable determination of pigment dispersion syndrome patients' risk of developing glaucoma, as well as adding to the understanding of the pathology of the disease.

Project Description

Additional Personnel

Marvin Podgor	M.S.	Statistician, Biometry and Epidemiology Program, NEI

Clinical Protocol Number

76-EI-189

Objectives

This project was designed (1) to compare patients with and without glaucoma who have pigment dispersion by documenting and following the clinical features and courses of disease and by evaluating performance on a variety of diagnostic tests; (2) to determine the presence of abnormal aqueous humor dynamics in glaucoma and nonglaucoma patients with pigmentary dispersion; and (3) to compare the association of pigment dispersion, with and without glaucoma, with possible genetic markers.

Methods

A complete evaluation includes the following: complete family history with detailed pedigree; best-corrected visual acuity with manifest refraction; slit-lamp biomicroscopy; visual field examination (Goldmann I_2e and I_4e); applanation Goldmann tension; photography of iris color, iris transillumination, and Krukenberg spindle; A-scan, anterior chamber depth, and anterior chamber volume measurements; goniophotography; static perimetry; baseline tonography and water-drinking tonography 1 hour later, when indicated; fasting blood sugar, when indicated; dilated ophthalmoscopic examination (using 2.5% phenylephrine and 1% cyclogel); and stereophotographs of the optic nervehead.

Major Findings

One hundred sixty-four patients were classified into three groups: (1) pigment dispersion syndrome (PDS) without abnormal ocular pressure, (2) PDS with ocular hypertension, and (3) PDS with glaucoma (PDS+GL). Analysis of baseline characteristics with respect to anatomical and physiological parameters has yielded the following conclusions:

1. It appears that the majority of patients recruited have PDS with a benign course: They do not develop ocular hypertension or glaucoma.

2. Consequently, family members of PDS patients should be alerted and appropriately screened. PDS may be familial and can show a dominant inheritance pattern.

3. Analyses of graded iris transillumination, the amount of pigment deposited on the trabecular meshwork, and the anterior chamber depth have demonstrated no significant differences among the three categories of PDS. Thus, pigment deposited in the angle may be only a secondary factor adversely affecting an already compromised outflow facility that is primarily a result of open-angle glaucoma.

4. It also appears that those patients who develop ocular hypertension and demonstrate early field changes can be managed medically by control of intraocular pressure and reversal of early field loss. Patients who develop glaucoma do not appear to be more difficult to treat than patients with open-angle glaucoma.

5. The phenomenon of unilateral or asymmetric PDS, in which there is little difference between the measurements of the two eyes, is being investigated in followup studies.

6. In our series, retinal detachment does not appear to occur with any greater frequency than with high myopia. A history of asymptomatic and nonprogressive peripheral retinal holes was noted in two patients.

7. The data from this study have been computerized, and an indepth analysis is under way.

Significance to Biomedical Research and the Program of the Institute

These results may facilitate determination of the risk of the development of glaucoma for patients with pigment dispersion. Specifically, it may be possible to identify which features have predictive value in forecasting which PDS patients will develop visual field defects. In addition, the data will aid investigation of the relationship of "pigmentary" glaucoma to the known characteristics of open-angle glaucoma.

Proposed Course

This project will be continued for 3 more years to obtain additional data on the patients enrolled in the study and to recruit more patients to add to the knowledge about PDS.

NEI Research Program

Glaucoma—Other Glaucomas (Developmental, Congenital, and Infantile Glaucomas)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00060-15 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Visual Function and Ocular Pigmentation in Albinism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	Lessie McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
	Evrydiki Bouzas	M.D.	Visiting Scientist	OGCSB, NEI
	Malina Drews-Bankiewicz	M.D.	Visiting Associate	OGCSB, NEI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.31	0.26	0.05

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [X] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with hypomelanotic disorders, such as ocular albinism, oculocutaneous albinism, Chediak-Higashi disease, Hermansky-Pudlak syndrome, and iris transillumination defects, are being recruited to determine visual function with these conditions and to evaluate the changes in visual function over time. Family members are evaluated in an attempt to determine factors which may identify the heterozygous state.

Project Description

Clinical Protocol Number

76-EI-207

Objectives

The objectives of this study are (1) to relate the level of visual function to the amount of ocular pigmentation, especially iris and retinal pigmentation; (2) to correlate the amount of nystagmus with visual acuity and iris pigmentation; (3) to determine whether ocular pigmentation, visual acuity, and nystagmus change with age; (4) to identify the heterozygous state of family members; and (5) to determine whether abnormalities of crossing of the optic nerve fibers can be correlated with the lack of pigmentation and whether previous reports of crossing abnormalities can be confirmed.

Methods

For each patient, a complete family history with detailed pedigree is compiled and the following procedures are performed: best-corrected visual acuity near and at a distance with refraction; slit-lamp examination; psychophysical testing, including D-15 and Munsell 100 hue as well as rod and cone thresholds; dilated ophthalmoscopic examination; photography to document hair color, eye color, iris transillumination, and the status of the disc and macula; visually evoked response testing; and, in selected patients, contrast sensitivity measurements. Information on family members is collected by examination of best-corrected visual acuity, slit-lamp examination of iris, photography of iris transillumination, and fundus examination when vision is not corrected to 20/20.

Major Findings

Major findings were as follows:

1. Examination of 82 patients and family members indicated that transillumination of the iris may occur in the absence of recognized albinism. The pattern, which appears to be punctate, may be present in a diffuse manner or limited to the 6 o'clock sector. The finding is not associated with nystagmus.

2. These patients presented with marked iris transillumination, reduced pigmentation of the fundus, and no nystagmus, but they had decreased visual acuity, which has improved in conjunction with an increase in the pigmentation of the fundae.

3. Visually evoked responses were normal in some patients, but in a subset of albinos, there was evidence of nonceniform pattern of asymmetry due to the miswiring of the visual pathways. The low amplitude of the visually evoked potentials recorded in a consecutive series of patients shows the difficulties of studying the phenomenon in a clinical setting.

Significance to Biomedical Research and the Program of the Institute

These data may allow identification of the carrier state of albinism, which would be important in genetic counseling. Determination of whether the development of the fovea is abnormal in albinism, whether this abnormal foveal development is the cause of the decreased visual acuity in albinism, or, alternatively, whether decreased visual acuity is secondary to hypopigmentation and the resultant light scatter and glare may be possible. Collection of these data also will facilitate ascertainment of whether visual acuity improves with age and whether this correlates with changes in pigmentation.

In addition, studies are being conducted to verify the reported findings of abnormalities of the crossing fibers, as measured by visually evoked responses, contrast sensitivity, degree of nystagmus, and amount of pigmentation.

Proposed Course

This project will be continued for 5 more years to obtain additional data.

NEI Research Program

Retinal and Choroidal Diseases—Developmental and Hereditary Disorders

Publications

Bouzas EA, Caruso RC, Drews-Bankiewicz MA, Kaiser-Kupfer MI: Evoked potential analysis of visual pathways in human albinism. *Ophthalmology*, submitted.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00083-16 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gyrate Atrophy of the Choroid and Retina and Other Retinal Degenerations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	Evrydiki Bouzas	M.D.	Visiting Scientist	OGCSB, NEI
	Lessie McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
	Pushpa K. Sran	M.D.	Medical Officer	OGCSB, NEI
	Doris Collie	A.A.	Ophthalmic Technician	OGCSB, NEI

COOPERATING UNITS *(if any)*

Howard Hughes Medical Institute Laboratory and Department of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, MD (David L. Valle, M.D.)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.15	0.75	0.40

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [X] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with gyrate atrophy of the choroid and retina are examined systematically to confirm the diagnosis. Skin fibroblasts from affected patients and family members are grown in tissue culture and assayed for ornithine aminotransferase activity. The results are evaluated for correlation with the presence of homozygosity or heterozygosity for the disease trait. Each patient is given a trial of pyridoxine to see whether serum concentration of ornithine can be reduced; if so, the patient is classified as a "responder," and treatment with pyridoxine is continued. Nonresponder and responder patients are then placed on a low-arginine, low-protein diet with supplemental amino acids and observed for arrest or improvement of the disease. If patients are not considered eligible for the diet, or if they appear unable to comply with the dietary regimen, we follow them to record the natural progression of the condition. Patients with other forms of retinal degeneration, such as retinitis pigmentosa, fundus flavimaculatus, juvenile retinoschisis, and Usher's syndrome, also are examined. The courses of their diseases are compared with those of gyrate atrophy patients.

Project Description

Additional Personnel

Laura Wozencraft	M.S.	Genetic Counselor, OGCSB, NEI
J. Fielding Hejtmancik	Ph.D.	Medical Officer, LMOD, NEI
Susan Gentleman	Ph.D.	Biologist, LRCMB, NEI

Clinical Protocol Number

78 EI-01

Objectives

This project is being conducted (1) to determine the biochemical processes responsible for the elevated plasma ornithine and the chorioretinal lesions that occur in gyrate atrophy (GA) of the choroid and retina; (2) to determine which patients respond to pyridoxine treatment with a decrease in plasma ornithine concentration; (3) to determine whether treating "responders" with pyridoxine and nonresponders with an arginine-deficient diet will arrest the progress of chorioretinal atrophy; (4) to study the natural history of this condition when intervention is not undertaken and to determine the degree of heterogeneity; (5) to define the molecular mutations and compare the molecular defect with the clinical features of the disease; and (6) to characterize and follow the progression of lens opacities, obtaining lens specimens at the time of cataract extraction for protein analysis.

Methods

Patients suspected of having GA of the choroid and retina are examined according to a standard set of procedures to confirm the diagnosis. Plasma ornithine concentration is measured periodically. Punch biopsies of the skin are grown in tissue culture, their ornithine aminotransferase activity is measured, and patient molecular defect is characterized. Complete evaluation of ocular function in these patients includes best-corrected visual acuity, Goldmann visual fields, color vision, cone thresholds, dark adaptation, electroretinogram (ERG), foveal electroretinogram (FERG), electrooculogram (EOG), contrast sensitivity, and Stiles-Crawford effect.

Major Findings

Gyrate atrophy (GA), a rare autosomal recessive disorder, is associated with hyperornithinemia, overflow ornithinuria, and a deficiency of activity of the mitochondrial enzyme ornithine-δ-aminotransferase (OAT). Although rare, the condition has been described worldwide in all races. Thirty-six patients have been recruited and evaluated in this study. The patients' ethnic origins vary and include African-American, Asian Indian, English, Finnish, German, Israeli, Lebanese, Portuguese, Scottish, Turkish, and Welsh.

In this study, among 44 patients, 22 females and 22 males range in age from 2.5 to 65 years, with 10 children less than age 12 at the time of recruitment. Observations of these patients have enabled documentation of both clinical evidence and laboratory heterogeneity.

Analysis of the mutation that causes GA of the choroid and retina has been undertaken by Drs. David Valle and Grant Mitchell and colleagues of The Johns Hopkins University. They have analyzed probands from 72 GA pedigrees. No gross structural alterations of the OAT gene have been detected; 85% of the probands express nearly normal amounts of normal-sized OAT mRNA. The remainder express little or no OAT mRNA (n = 5) or an mRNA with an altered size (n = 2). Western blot studies showed the OAT antigen to be absent in 67% of the mRNA+ mutants and all of the mRNA– mutants. A total of 14 mutations have been delineated at the molecular level: 10 missense mutations (M1I, R180T, L402P, C93F, Y55H, R154L, A270P, R271KL, G375V, and P417L/L437F); a single nucleotide deletion at cDNA position +159 (H53fs); an interesting in-frame three-nucleotide deletion of Ala-184 (A185F0), and a nonsense mutation at a CpG dinucleotide (R396ter).

The functional consequences of several mutations have been examined by substituting the mutations into otherwise wild-type OAT cDNA in the expression vector P91023b and transfecting the recombinant constructs into CHO-K1 cells that lack endogenous OAT mRNA or protein. Three (R180T, L402P, A184D0) have been shown to encode a CRM+, enzymatically inactive protein, while M1I—as expected for an initiation codon alteration—has a CRM– phenotype. Studies are under way to correlate mutational heterogeneity with clinical and biochemical heterogeneity.

The earliest clinical and electrophysiologic features were documented in the two youngest patients (ages 2.5 and 3 years). The minimal evidence of clinical retinal changes when significant reduction of rod and cone function is seen by electroretinographic studies is noteworthy.

Clinical and biochemical evidence of genetic heterogeneity is present in these patients. Fewer than 10% of patients have been reported to have a 30-50% decrease in plasma ornithine following treatment with vitamin B_6. Only one of our patients showed an *in vivo* response to this treatment. Comparisons of sibships reveal that there is a greater degree of interfamilial variability than intrafamilial variability.

Whereas arginine is the precursor of ornithine in the metabolic pathway of ornithine metabolism, we have undertaken a dietary intervention study limiting arginine. Of 25 patients placed on a low-protein (i.e., low arginine) diet, all sustained significant reduction of ornithine during hospitalization; however, the diet was discontinued in 4 Finnish patients following their discharge because of poor compliance and in 7 other patients because of a variety of factors. Of 15 patients remaining on the diet, 4 have excellent control; 4, fair control; and 4, erratic control. One young child was followed for too short a period of time to assess control. Ophthalmologic evaluations are performed on all patients every 6 to 12 months, travel permitting.

In the two patients with the best biochemical control for the longest time (11 and 12 years old, respectively), there was evidence of improved visual function. One patient, after being on the diet for 14 months, showed improved dark adaptation and average ERG and color vision. This improvement was sustained for 30 months, then the ERG amplitude showed a small but definite reduction. The second patient who had lowered plasma ornithine levels and who had been on the diet for 11 years showed progressive improvement in visual field and color vision and has since remained stable. A third patient, despite fair control, was stable for 36 months but has deteriorated for the past 18 months. It should be noted that she was the oldest patient and had the most advanced disease at the outset. Other patients followed for various periods of time currently appear stable. Of particular interest are the children who were ages 2.5 to 9 years old at the outset of diet. The results indicate that as a result of dietary intervention the course of the disease in the younger of each sibship has been improved, compared with that of the older sibling.

All but one patient over age 11 have had progressive cataracts in the posterior capsule. They present a uniform histologic picture and can be identified by their characteristic pattern in image analysis.

Significance to Biomedical Research and the Program of the Institute

GA of the choroid and retina is the first isolated of the genetically determined severe retinal degenerations for which a specific biochemical marker and concomitant enzyme defect have been demonstrated. Designed to test the efficacy of treatment for this blinding eye disease, this study will serve as a model for the investigation of other genetically determined retinal degenerations. Study of the two young patients is the best opportunity for the evaluation of diet control. This disease is a likely candidate for future studies to begin gene therapy.

Proposed Course

This project will be continued for 3 more years to assess further the knowledge concerning the reduction of ornithine to halt chorioretinal degeneration.

NEI Research Program

Retinal and Choroidal Disease—Development and Hereditary Disorders

Publications

Brody LC, Mitchell GA, Obie C, Michaud J, Steel G, Fontaine G, Robert M-F, Sipila I, Kaiser-Kupfer M, Valle D: Ornithine σ-aminotransferase mutations in gyrate atrophy. *J Biol Chem* 267:3302-3307, 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00163-11 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

NIH Interinstitute Genetics Program: The Genetics Clinic

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	Evrydiki Bouzas	M.D.	Visiting Scientist	OGCSB, NEI
	Mark Scott	M.D.	Senior Staff Fellow	OGCSB, NEI
	Lessie McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Anren Li	M.D.	Visiting Associate	OGCSB, NEI
	Laura Wozencraft	M.S.	Genetic Counselor	OGCSB, NEI

COOPERATING UNITS *(if any)*

Interinstitute Medical Genetics Program, NIH

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.8	0.3	0.5

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [X] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Interinstitute Genetics Program and the Genetics Clinic supported by the Clinical Center offer a multidisciplinary approach to patients with genetic disease (Z01 CP 05139-06 CEB). Involved in the program are researchers from all Institutes. Patients evaluated in the clinic represent a broad spectrum of genetic diseases. During the past year, approximately 200 persons seen represented about 60 distinct disease categories. Due to the high frequency of ocular involvement in many of the cases, almost all the patients were evaluated by Clinical Branch staff or were discussed in consultation. The clinic serves as a source of interesting case material concerning patients with inherited or developmental abnormalities of the visual system.

Project Description

Clinical Protocol Number

Interinstitute Medical Genetics Program

Objectives

The objectives of this Program are (1) to evaluate patients with ocular abnormalities associated with genetic disease in the context of a multidisciplinary approach to the patient; (2) to provide genetic counseling to patients at risk for inherited ocular disease; (3) to recommend and advise appropriate evaluation for the ocular problem; and (4) to provide training in the diagnosis, counseling, and treatment of individuals with or at risk for genetic disease, as well as in the research approach to genetic disease.

Methods

Referred patients are examined, and the appropriate diagnostic ophthalmologic workup is recommended.

Major Findings

1. Iris nodules were seen commonly in the classic cases of neurofibromatosis (NF1) and less frequently seen in patients with less-well-defined disease. They were seen rarely in patients with bilateral acoustic neuroma (BAN or NF2). Interestingly, in a series of 14 consecutive patients with Cushing's disease, two patients (14%) had typical, unilateral lisch nodules. To our knowledge, the association of NF1 on lisch nodules with Cushing's disease has not been described. The association of Cushing's disease and lisch nodules may represent a mild form of multiple endocrine neoplasia of the mixed type. It is possible that a common underlying mechanism leads to the overgrowth of melanocytes in the iris and corticotrophs in the pituitary. Patients with NF2 showed increased frequency of posterior capsular cataracts, which serve as an excellent marker, being present in 80% of individuals with NF2. A new finding is the association of peripheral cortical cataracts in 37.8% of NF2 patients. In a group of severely affected NF2 patients, it appears that combined pigment epithelial and retinal hamartomas are also an ocular marker for NF2. In fact, there may be a predilection for the macula in some cases.

2. Serious ocular complications were observed in 13 long-term postrenal transplantation nephropathic cystinosis patients. These complications included decreased visual acuity and visual function, as measured by psychophysical and electrodiagnostic tests, band keratopathy, and posterior synechia. Corneal transplantation may be necessary in cases with debilitating symptoms from recurrent erosion after all other treatment modalities have failed. In two such patients, the corneal grafts have remained clear for as long as 6 years.

3. Ophthalmic studies performed in a population of patients with endogenous Cushing's syndrome revealed that posterior subcapsular cataracts were an infrequent phenomenon compared with exogenous Cushing's syndrome. Although an uncommon finding, central serous chorioretinopathy was seen in 3 of 60 patients (5%), suggesting that glucocorticoids may play a role in the development of the disease.

Significance to Biomedical Research and the Program of the Institute

Genetic and developmental anomalies of the eye are a major cause of blindness and visual disability; they are responsible for about 35% of the cases of blindness in developed nations. Involvement with the Interinstitute Genetics Program affords a systematic approach to studying these and other conditions associated with genetic diseases.

Proposed Course

The project is in a growth phase and will be expanding in future years.

NEI Research Program

Retinal and Choroidal Disease—Development and Hereditary Disorders

Publications

Bouzas EA, Kransewich D, Koutroumanidis M, Papadimitriou A, Marini JC, Kaiser-Kupfer MI: Ophthalmological examination in the diagnosis of Proteus syndrome. *Ophthalmology* 100:334-338, 1993.

Bouzas EA, Freidlin V, Parry DM, Eldridge R, Kaiser-Kupfer MI: Lens opacities in neurofibromatosis 2: Further significant correlation. *Br J Ophthalmol* 77:354-357, 1993.

Bouzas EA, Mastorakos G, Chrousos GP, Kaiser-Kupfer MI: Lisch nodules in Cushing disease. *Arch Ophthalmol* 111:439, 1993.

Bouzas EA, Mastorakos GM, Chrousos GP, Kaiser-Kupfer MI: Posterior subcapsular cataract is infrequent in endogenous Cushing syndrome. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1064, 1993.

Bouzas EA, Mastorakos G, Friedmann T, Scott MI, Chrousos GP, Kaiser-Kupfer MI: Posterior subcapsular cataracts in endogenous Cusing Syndrome: An uncommon manifestation. *Invest Ophthalmol Vis Sci* 34:3497-3500, 1993.

Bouzas EA, Parry DM, Eldridge R, Kaiser-Kupfer MI: Visual impairment in patients with neurofibromatosis 2. *Neurology* 43:22-623, 1993.

Bouzas EA, Scott MH, Mastorakos GP, Chrousos GP, Kaiser-Kupfer MI: Central serous chorioretinopathy in endogenous hypercortisolism. *Arch Ophthalmol*, 111: 1229-1233, 1993.

Kaiser-Kupfer MI, Bouzas EA: Ocular manifestations of metabolic disorders. *Curr Opin Ophthalmol* 3:221-227, 1992.

Mastorakos G, Bouzas EA, Burnier MN, Chrousos GP, Chrousos GA: Presence of immunoreactive corticotropin releasing hormone in the optic nerve but not the inflammatory infiltrate of allergic optic neuritis/encephalomyelitis. *Invest Ophthalmol Vis Sci* 34(4)(suppl):1000, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00211-08 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

A Double-Masked Controlled Randomized Clinical Trial of Topical Cysteamine

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	Lessie McCain	R.N.	Nurse Specialist	OGCSB, NEI
	Manuel Datiles	M.D.	Medical Officer	OGCSB, NEI
	Evrydiki Bouzas	M.D.	Visiting Scientist	OGCSB, NEI
	Mark Scott	M.D.	Senior Staff Fellow	OGCSB, NEI
	Anren Li	M.D.	Visiting Associate	OGCSB, NEI

COOPERATING UNITS *(if any)*

Human Genetics Branch, National Institute of Child Health and Human Development; NIH, Bethesda, MD (William Gahl, M.D., Ph.D.)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.70	0.45	0.25

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [x] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Nephropathic cystinosis is an autosomal, recessively inherited storage disease in which nonprotein cystine accumulates within cellular lysosomes due to a defect in lysosomal cystine transport. Ocular manifestations include photophobia; crystal deposits in the cornea, conjunctiva, and iris; and depigmentation of the retina.

Ten years ago, cysteamine, a free thiol that depletes cystine from cells, was introduced in the therapy of cystinotic patients. Although patients had improved growth and stabilized renal function, there was no noticeable effect on the accumulation of corneal crystals. Recent studies showed that corneal cells in tissue culture are readily depleted of cystine by the introduction of cysteamine, making feasible the use of topical ophthalmic cysteamine to circumvent the humoral route. After appropriate animal studies to test for complications revealed none, we began a double-masked clinical trial to test the efficacy of topical cysteamine (0.1% and 0.5%) in humans.

To date, in 14 of 29 young patients the code was successfully broken; of the 15 remaining, 2 died, 1 discontinued medication, and 12 are still in the trial with poor compliance and have not been seen for followup. Because of the success in the younger patients, this study was expanded to include older patients, 3 to 31 years of age. The findings have been most exciting: Twenty-three patients have shown a significant decrease in crystals in treated eyes as well as improvements in comfort, i.e., relief of pain and photophobia. This study has resulted in significantly improved quality of life for the successfully treated patients. Because of the success of this clinical trial, and evidence from the cysteamine-benzalkonium trial (Protocol Number 93 EI-0230), the Food and Drug Administration has requested that all patients in this protocol be switched to cysteamine plus benzalkonium and receive medication in both eyes. Each patient then will be judged by a comparison with his or her own natural history.

Project Description

Additional Personnel

Ernest M. Kuehl	Chief, Photography Section, OGCSB, NEI

Clinical Protocol Number

86-EI-62

Objectives

The purpose of this project is to test the efficacy of topical cysteamine in patients with nephropathic cystinosis.

Methods

Slit-lamp examination and photography of the cornea are performed by a masked observer to determine whether there is a difference in the quantity of crystals seen in the cornea.

Major Findings

Topical cysteamine eyedrops (0.5%) are well tolerated. The crystal accumulation is reversible in very young patients, who do not have crystals packing the cornea, as well as in older patients in which the crystals pack the cornea.

Significance to Biomedical Research and the Program of the Institute

The continued accumulation of crystals in the cornea appears to lead to increasing discomfort in cystinosis patients, who develop severe photophobia with recurrent corneal erosions. Topical cysteamine treatment, which has been found to halt the process, has led to an improvement in the quality of life of these patients.

Proposed Course

This study will be replaced by a study in which the crystal accumulation will be compared with the natural history of the condition.

NEI Research Program

Corneal Diseases—Ocular Surface Problems (Drug Delivery and Toxicity)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00282-01 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Usher's Syndrome—Clinical and Molecular Studies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI
Others:	J. Fielding Hejtmancik	M.D., Ph.D.	Medical Officer	LMOD, NEI
	Mark H. Scott	M.D.	Senior Staff Fellow	OGCSB, NEI
	Rafael C. Caruso	M.D.	Visiting Scientist	OGCSB, NEI
	Laura A. Wozencraft	M.S.	Genetic Counselor	OGCSB, NEI
	Anita Pikus	M.A.	Chief, Audiology Unit	HS/NOB, NIH

COOPERATING UNITS *(if any)*

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [X] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to document the clinical features of Usher's syndrome, to refine the localization, and eventually to isolate the genes causing this disease.

Project Description

Clinical Protocol Number

93 EI-0161

Objectives

The objectives of this study are (1) to relate the level of visual function to the amount of ocular pigmentation, especially iris and retinal pigmentation; (2) to correlate the amount of nystagmus with visual acuity and iris pigmentation; (3) to determine whether ocular pigmentation, visual acuity, and nystagmus change with age; (4) to identify the heterozygous state of family members; and (5) to determine whether abnormalities of crossing of the optic nerve fibers can be correlated with the lack of pigmentation and whether previous reports in abnormalities of crossing can be confirmed.

Methods

Included in the evaluation will be audiometric, vestibular, ophthalmologic, and electrophysiologic and electrodiagnostic testing. These clinical findings will help classify the features of the different types of Usher's syndrome, as well as correlate the phenotypic features with the genetic mutation. To identify the genetic mutation, we will study informative families, collecting blood specimens from all available family members for studies that will utilize molecular technology developed for linkage analysis. In cases in which there are no other affected family members, blood specimens will be obtained to study specific gene mutations when the specific gene or genes for Usher's syndrome are identifiable.

Major Findings

The recruitment for this project has begun: 40 patients have been recruited. Patients are being evaluated, and their blood specimens are being collected and maintained in the laboratory. Linkage analysis on these families has not yet begun.

Significance to Biomedical Research and the Program of the Institute

By molecular studies of patients with Usher's syndrome, mutations may be correlated with clinical findings and genes responsible for Usher's syndrome may be defined, leading to the possibility of genetic therapy at some point.

Proposed Course

Patient recruitment into the study will be continued.

NEI Research Program

Retinal and Choroidal Disease—Development and Hereditary Disorders

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00283-01 OGCSB

PERIOD COVERED

July 13, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

A Double-Masked Controlled Randomized Clinical Trial of Topical Cysteamine

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Muriel I. Kaiser-Kupfer M.D. Chief OGCSB, NEI

COOPERATING UNITS *(if any)*

Human Genetics Branch, National Institute of Child Health and Human Development, NIH (William A. Gahl, M.D., Ph.D.)

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [X] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cysteamine ophthalmic drops prepared for commercial availability must pose no risk for contamination and subsequent infection. This study is designed to demonstrate conclusively that benzalkonium chloride plus cysteamine is a safe preparation that is effective when administered every waking hour to patients who have nephropathic cystinosis in corneal crystals.

Project Description

Clinical Protocol Number

93 EI-0230

Objectives

The purposes of this study are to determine whether the addition of benzalkonium chloride to cysteamine eyedrops is a safe preparation and whether this preparation is effective in removing crystals from patients with cystinosis.

Methods

Thirty patients were to be entered into this study. These were nephropathic cystinosis patients for whom the code had been successfully broken in conjunction with protocol 86 EI-62. Each patient was randomized, with one eye always serving as a comparison to the fellow eye. One eye was treated with cysteamine alone; the second, with cysteamine 0.5% plus benzalkonium 0.101%. The primary outcome parameter was the safety of the additive benzalkonium. There were periodic checks of retinas for irritation attributable to benzalkonium. Efficacy for cysteamine was evaluated over a 6-month period.

Major Findings

In the 20 patients who have been enrolled in this protocol so far, there is no evidence of toxicity from the addition of benzalkonium to the cysteamine eye drops. Furthermore, if used as required, the cysteamine plus benzalkonium appears to be as effective as the cysteamine without benzalkonium.

Significance to Biomedical Research and the Program of the Institute

Ensuring that benzalkonium added to cysteamine eyedrops is not toxic but still effective will move this drug one step closer to new drug approval by the FDA.

Proposed Course

Since the toxicity has been proven to be nil and the drug is still effective, this protocol will be terminated.

NEI Research Program

Corneal Diseases—Ocular Surface Problems (Drug Delivery and Toxicity)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 EY 00284-01 OGCSB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characteristics of Macular Scotomas in Patients With Primary Monofixation Syndrome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Mark H. Scott	M.D.	Senior Staff Fellow	OGCSB, NEI
Others:	Rafael Caruso	M.D.	Visiting Scientist	OGCSB, NEI
	Muriel I. Kaiser-Kupfer	M.D.	Chief	OGCSB, NEI

COOPERATING UNITS *(if any)*

Marshall M. Parks, M.D. (Private Practice), Washington, DC

LAB/BRANCH

Ophthalmic Genetics and Clinical Services Branch

SECTION

Section on Ophthalmic Genetics

INSTITUTE AND LOCATION

NEI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.175	0.175	0.0

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [X] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The monofixation syndrome (MFS) is a defective form of binocular vision characterized by preservation of extramacular function with absence of macular fusion. Fusion is defined as the ability to perceive simultaneously similar images projected onto corresponding areas of each retina. The fusing of images is a binocular phenomenon that occurs in the higher-order parastriate and peristriate areas of the prestriate visuomotor cortex (Brodmann areas 18 and 19, respectively). Patients in this protocol have been examined by Goldmann perimetry and the Lancaster red-green test to map the facultative macular scotoma in the nonfixating eyes in patients with primary MFS, surgically corrected congenital esotropia, and anisometropic amblyopia. The characteristics of the scotomas in each population of patients will be compared. The results of this study will contribute to the understanding of primary MFS by testing the hypothesis that primary MFS is a mild expression of a gene or series of genes that causes congenital esotropia and that these genes exert their variable expression on the binocular neurons of the central macular fusion area.

Project Description

Clinical Protocol Number

93 EI-0067

Objectives

The objectives of this study are to plot the characteristics of macular scotomas in patients with primary monofixation syndrome (MFS) and to gain data to test the hypothesis that such MFS scotomas may result from expression of a particular gene or series of genes that cause congenital esotropia.

Methods

Patients entering the study undergo a complete ophthalmic examination by Goldmann perimetry. By use of the Lancaster red-green test, the facultative macular scotoma is mapped out in the nonfixating eyes of patients with primary MFS, surgically corrected congenital esotropia, and anisometropic amblyopia. The kinetic mode of the perimeter is used to plot the size and shape of the scotoma; the static mode of the perimeter is used to determine the depth of suppression within the scotomatous region. Lancaster red-green tests both standard and automated versions also are used to plot the size of the scotomas. The characteristics of the scotomas in each population of patients can then be compared with each other.

Major Findings

Although the study is in its preliminary phases, it has been possible to plot the scotomas as planned. Analysis of the data awaits further recruitment.

Significance to Biomedical Research and the Program of the Institute

A better understanding of mechanisms of development of scotomata will help in elucidating the etiologies of certain potential blinding conditions such as amblyopia.

Proposed Course

Work will continue with the completion of the analysis of data from patients who have been recruited into the study.

NEI Research Program

Developmental and Strabismus

Index

Index